ELEGEN

APPLICATION NOTE

Validation of ENFINIA Plasmid DNA Production

SUMMARY

Plasmids are essential tools in molecular biology, enabling applications such as heterologous gene expression, genome engineering, and viral production. However, the production of synthetic plasmid DNA, i.e. clonal gene synthesis, with long or highly complex sequences faces several challenges, including inefficiencies, high error rates, and lengthy workflows. To address these limitations, Elegen developed ENFINIA™ Plasmid DNA. This high-fidelity clonal gene synthesis process leverages ENFINIA Linear DNA fragments with a low error rate (1 in 70,000 bp), enabling synthesis of gene sequences as long as 15 kb to ship as fast as 10 business days. In the following study, the performance of ENFINIA Plasmid DNA was validated using 107 distinct gene sequences across seven vector backbones, achieving over 92% sequence-perfect cloning success, even for gene lengths of up to 15 kb with high complexity. We also confirm that ENFINIA Plasmid DNA can successfully enable the expression of reporter genes in *E. coli* and in *in vitro* transcription-translation systems. Taken together, ENFINIA Plasmid DNA offers researchers a streamlined alternative to conventional molecular cloning and other, more limited clonal gene synthesis techniques.

Key Points

- ENFINIA Plasmid DNA achieves a 92% success rate of delivering sequence-perfect clones containing exceptionally complex genes up to 15 kb long.
- Leveraging long, accurate fragments produced with Elegen's patented cell-free cloning technology, ENFINIA Plasmid DNA is delivered NGS-verified in as fast as 10 business days.
- Functional validation of common reporter genes has been demonstrated in *E. coli* and TXTL expression system.

INTRODUCTION

Plasmids are small, circular pieces of extrachromosomal DNA used for various applications in the life sciences. Their modular nature and ability to replicate independently of chromosomal replication in host cells (typically bacteria) make them advantageous for heterologous gene expression, the production of live viruses, genome engineering, and other applications that necessitate the manipulation of heterologous fragments of DNA.

Several molecular cloning techniques are used to create plasmids for these various applications. In general, they involve a multi-step process to produce a cloned plasmid containing a sequence-perfect molecule.[1] Despite significant advancements in cloning techniques, such as Gibson and Golden Gate assembly, due to a reliance on host cell lines, cloning remains a major bottleneck in many areas of life science research.[2,3] As gene or construct lengths grow in size and complexity, cloning efficiency can decrease, and chances of errors within the gene sequence can increase, often limiting the benefits of downstream use. Traditionally, the synthesis of plasmids containing long genes (> 3 kb) requires the subcloning of short fragments, with high error rates (typically 1 error in every 3500 bp). Once all fragments are subcloned in, the fragments are assembled, cloned, and sequenced until a sequence-perfect clone is identified. This lengthy process is costly, error-prone, and typically takes weeks or months to build long and complex genes.

Elegen's ENFINIA Plasmid DNA technology accelerates gene synthesis by using high-fidelity ENFINIA Linear DNA fragments (up to 7 kb, 1 error per 70,000 bp on average) to bypass subcloning. The unique combination of length and accuracy enables the assembly of plasmids with fewer fragments that do not require subcloning. The efficiency gained by this enables the production and delivery of sequence-verified plasmids with inserts up to 15 kb in as little as 10 business days, significantly reducing turnaround time compared to traditional approaches.

In the following application note, we validate the performance specifications of ENFINIA Plasmid DNA using sequences of varying lengths, %GC content, structural elements, and repeats. As a proof of concept, we use ENFINIA Plasmid DNA to synthesize plasmids with mCherry, mNeonGreen, and Purple WT reporter gene inserts and find that they are functional in *E. Coli* and also *in vitro* transcription-translation (TXTL) expression systems. These results support using ENFINIA Plasmid DNA for synthesizing functional plasmid DNA with nearly any gene sequence, reducing the time required for molecular cloning and other gene synthesis workflows.

MATERIALS AND METHODS

Performance Assessment of ENFINIA Plasmid DNA Production

Elegen's production of ENFINIA Plasmid DNA was validated using a test sequence panel composed of 107 distinct sequences sourced from the Addgene public database and beta-test customers. Additionally, the panel included modified sequences to evaluate different assembly factors. Overall, the selected sequences span the whole range of ENFINIA Plasmid DNA gene sequence acceptance criteria ***(Table 1)***. Sequences were cloned in one of seven vector backbones offered through Elegen, including pAAV2-Amp, pAAV2-CAG-Amp, pAAV2-CMV-Amp, pAAV2-hSyn-Amp, pBR322-Amp, psaRNA-Amp, and pUC19-Amp. Following the selection on antibiotic-containing plates, six clones were typically picked for screening, sequencing, and confirmation of plasmid identity.

	Standard Complexity	High Complexity
DNA Sequence Length	**300** – **15,000** bp	**300** – **15,000** bp
Overall GC Content	**25** – **65%**	**20** – **80%**
100 bp GC Content	**22** – **75%**	**12** – **85%**
Local GC Variation	Up to **60%**	Up to **70%**
Repeats	Up to **20** bp	Up to **150** bp
Homopolymers*	Up to **7** bases (G/C) Up to **8** bases (A/T)	Up to **8** bases (G/C) **12** bases (A/T)

Table 1. ENFINIA Plasmids Submission Acceptance Criteria and Specs

1. *ENFINIA Plasmid DNA can be ordered at a standard synthesis yield of 50 ng to 1 µg†, depending on the vector selected.*
2. *ENFINIA Plasmid DNA is delivered as dried-down DNA in a 96-well microplate, one plasmid per well.*
3. *Each ENFINIA Plasmid DNA sequence is NGS-verified before shipment.*
4. *Insert sequences should not contain elements that are toxic to E.coli.*

** At this time, the QC methods we use for production do not provide high confidence in the fidelity of A/T homopolymer sequences longer than 12 bp and G/C homopolymer sequences longer than 8 bp. We are working to address this in the near future.*

† Synthesis yield as measured by Thermo Fisher Scientific Quant IT Assay.

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

Three reporter genes, mCherry, Purple WT, and two codon-optimized mNeon Green (GFP-like reporter) variants, were designed *in silico* with the required template elements to enable use as plasmid templates for cell-free protein expression reactions (e.g., 5′ leader sequence, T7 promoter, terminator, RBS, 3′ trailer sequence, etc.), according to the instructions for myTXTL® Pro Cell-Free Expression Kit manufactured by Daicel Arbor Biosciences. The three reporter genes were synthesized using ENFINIA Linear DNA and assembled using the Elegen Plasmid DNA process downstream of the T7 promoter in the high copy number cloning vector pUC19 with an ampicillin resistance marker.

Plasmids were transformed into *E. coli* 10-beta competent cells (NEB). Six colonies per gene were picked and cultured overnight in TB with antibiotics, and plasmids were purified for sequencing and downstream applications.

Plasmid yields were evaluated by QuantIT or Qubit quantitative assays, and purity was determined by UV absorbance and absorption wavelength ratios (ABS260/230 and ABS260/280) using a DeNovix DS-11 FX spectrophotometer/Fluorometer.

Preparation of Vendor Plasmid DNA

The sequences of mCherry and mNeonGreen (one codon-optimized version) were submitted for synthesis through an independent vendor (named Supplier A) portal. Upon receipt, they were subjected to the same methodology described below (see Functionality Testing subsection) for the ENFINIA Plasmid DNA.

Functionality Testing

Plasmids were transformed into *E. coli* BL21 (DE3) cells (One Shot™, Invitrogen) according to the manufacturer instructions. Colonies were recovered by plating on LB agar plates containing 100 ng/µL ampicillin. Transformants with mCherry and mNeonGreen expression plasmids were incubated at 30°C overnight. Visual inspection of the colonies was done the next day, and the constructs from Elegen and Supplier A were evaluated in parallel.

Cell-Free TXTL Reactions

ENFINIA Plasmid DNA concentrations for mCherry, mNeonGreen, and Purple WT were adjusted to 24 nM and incubated at a final reaction concentration of 5 nM, as suggested by myTXTL® Pro Cell-Free Expression Kit manufacturer. The T7 deGFP control plasmid, provided with the myTXTL® Pro Cell-Free Expression Kit, was used as a positive reaction control.

Reactions were set up according to the manufacturer's instructions in a total volume of 12 µL and incubated at 27°C overnight (16h) without agitation. The reactions were stopped by placing them on ice and then spinning down at 16,000 x g for 3 minutes.

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). Expression of Purple WT was measured using a DeNovix DS-11 FX spectrophotometer/ Fluorometer at a volume of 1 µL using an absorbance of 574nm.

RESULTS

Validating Performance Specifications for ENFINIA Plasmid DNA

We collected 107 distinct sequences from public and private sources to experimentally validate the ENFINIA Plasmid DNA process. These sequences ranged from standard to high complexity (as defined in ***Table 1)***, with varying insert sizes, overall and local GC content, and repeats to cover the relevant acceptance criteria.

Once inserts were synthesized and NGS-verified using Elegen's cell-free process, they were cloned into one of the seven vector backbones. Following transformation and selection on antibiotic-containing media, cloning success was assessed by picking colonies and confirming that they contained sequence-perfect constructs.

For 4 of the backbones available, a series of AAV2 vectors with different promoters (pAAV2-Amp, pAAV2-CAG-Amp, pAAC2-CMV-Amp, and pAAV2-hSyn-Amp), all of the clones picked were sequence-perfect (i.e., 100% success rate; *Table 2*). For our backbones typically used in molecular biology for subcloning, pUC19, and pBR322, we tested 36 and 26 sequences, respectively, resulting in over 90% success rate *(Table 2)*. Using our psaRNA-Amp backbone across nine distinct sequences resulted in an 88.9% success rate *(Table 2)*. Across all standard (n=81) or high complexity (n=26) sequences cloned, the success rate was greater than 92%, and the average turnaround time for delivery was 10.8 and 12.1 business days, respectively.

Backbone	Complexity	Total	% Success Rate
pAAV2-Amp	All	10	**100.0%**
pAAV2-CAG-Amp	All	7	**100.0%**
pAAV2-CMV-Amp	All	11	**100.0%**
pAAV2-hSyn-Amp	All	8	**100.0%**
pBR322-Amp	All	26	**92.3%**
psaRNA-Amp	All	9	**88.9%**
pUC19-Amp	All	36	**91.7%**

Backbone	Complexity	Total	% Success Rate	Avg. TAT (bd)
All	Standard	81	**95.1%**	**10.8**
All	High	26	**92.3%**	**12.1**

Table 2. Success rate for ENFINIA Plasmid DNA process across all backbones and complexities

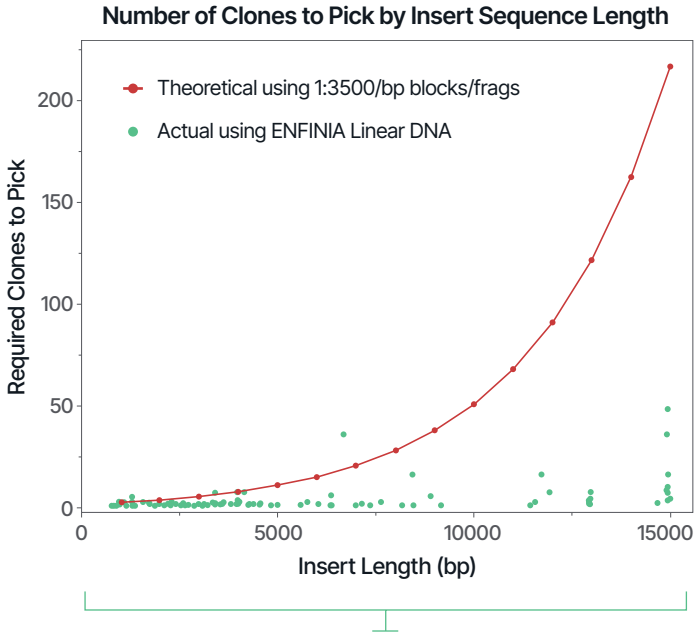

Figure 1. Robust ENFINIA Plasmid DNA production process for inserts up to 15 kb. ENFINIA linear inserts with low error rate of 1:70,000 per bp were cloned into vector backbones, transformed into E. coli, and plated on selective media. Colonies were picked and sequenced to identify sequence-perfect clones. Regardless of the insert length and backbone ordered, selection and sequencing of 6 colonies or less is sufficient to achieve a 95% synthesis success rate. Compared to standard gene fragments >5 kb with an error rate of 1:3500/bp blocks/frags, ENFINIA Plasmid DNA offers a significant advantage.

To further define the performance specifications of the ENFINIA Plasmid DNA process, we assessed whether insert length, %GC content, structural elements, and tandem repeat length impacted the number of clones that needed to be picked to select a sequence-perfect construct. In terms of insert length, results indicated that the process is robust up to 15 kb insert length, demonstrating the significant advantage over traditional clonal gene synthesis approaches that rely on shorter error-prone fragments *(Figure 1)*.

We also observed similar cloning success rates for inserts with overall GC content ranging from 20 - 80% and local GC content (per a 100 bp window) from 12% to 83% ***(Figure 2)***. The assembly process is also robust to secondary structures and repeats, including inverted terminal repeats (ITRs) ***(Figures 3A & 3B)***. Taken together, ENFINIA Plasmid DNA can accept a broad range of sequence lengths and identities, making it a powerful tool for many life science applications.

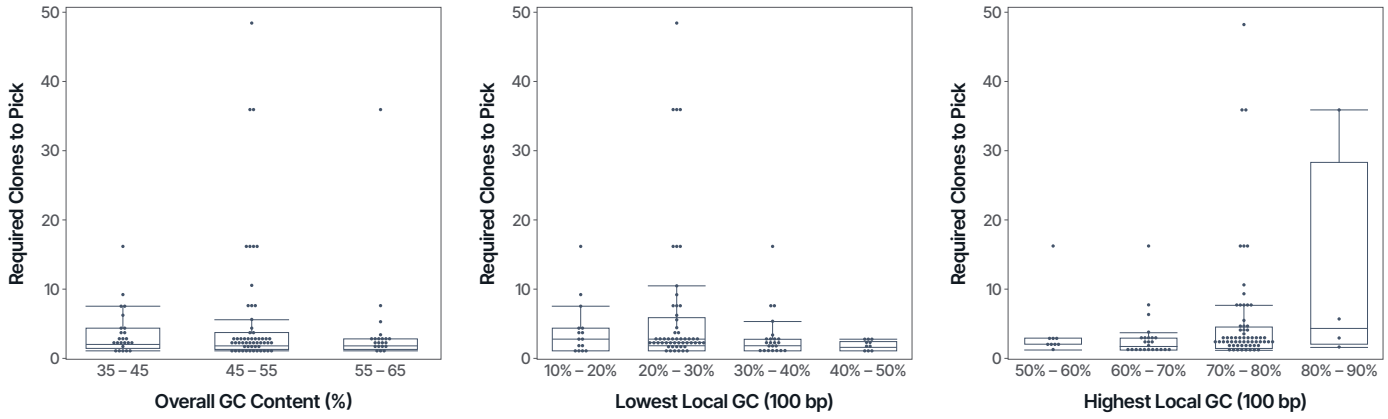

Figure 2. The ENFINIA Plasmid DNA process produces sequence-perfect clones, independent of overall and local GC content. ENFINIA Linear DNA inserts were cloned into vector backbones, transformed into E. coli and plated on selective media. Colonies were picked and sequenced to identify sequence-perfect clones. The number of colonies that needed to be picked post transformation was typically <6 and consistent across all overall and local GC content.

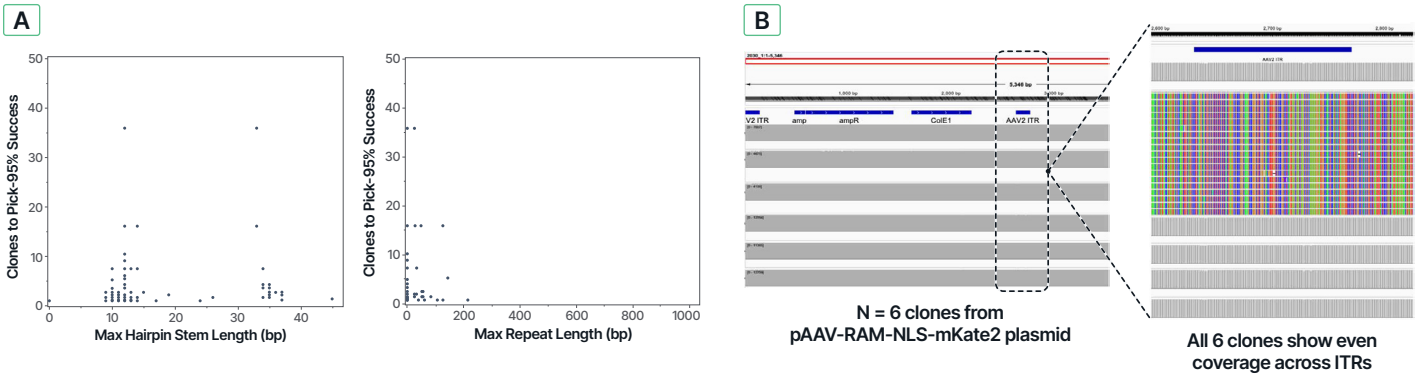

Figure 3. The ENFINIA Plasmid DNA process produces sequence-perfect clones, independent of the presence of structural elements.

A) *ENFINIA Linear DNA inserts were cloned into vector backbones, transformed into E. coli, and plated on selective media. Colonies were picked and sequenced to identify sequence-perfect clones. The number of colonies that needed to be picked post transformation was typically <6 and consistent across all overall and local GC content. Max repeat length is Calculated as the maximum length of sequence that is repeated >1 time with 95% sequence similarity for each plasmid insert.*

B) *Alignment (right panel) and coverage (left panel) of sequences from all 6 clones picked is shown.*

Functional Assessment of ENFINIA Plasmid DNA Using Cell-Free TXTL System

To investigate the utility of ENFINIA Plasmid DNA for heterologous gene expression, we synthesized reporter genes that are widely used for research ***(Table 3)***. Purple WT, mCherry, and mNeonGreen sequences were assembled using the ENFINIA plasmid process, and, in parallel, we outsourced synthesis to an external market leading clonal gene synthesis vendor, Supplier A, to serve as an experimental comparison ***(Table 3)***. Upon receipt, we transformed mCherry and mNeonGreen constructs into *E. coli*, and plated on antibiotic-containing media. Through visual assessment, we compared reporter gene expression from Elegen- vs. Supplier A-synthesized constructs and observed similar expression levels ***(Figure 4)***.

Gene	Gene size (bp)	Complexity	Vector backbone	
			Elegen	Supplier A
mCherry	1301	Standard	High copy number pUC19	High copy number pUC19
mNeonGreen (GFP-like)	835	Standard	High copy number pUC19	High copy number pUC19
Purple WT (non-fluorescent chromoprotein)	793	Standard	High copy number pUC19	N/A

Table 3. Reporter genes used in this study

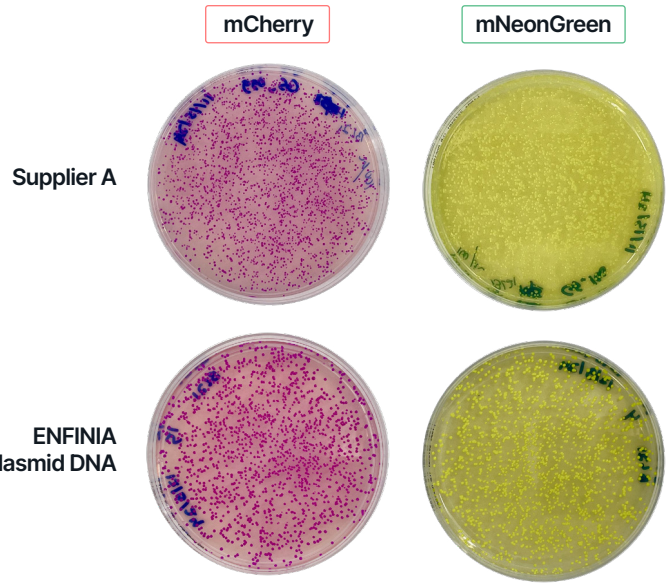

Figure 4. Comparable expression of mCherry and mNeonGreen reporter gene in E. coli host cells. mCherry (1.3 kb) and mNeonGreen (0.84 kb) synthesized by Elegen and by an external vendor were transformed into E. coli BL21 (DE3) and plated on selective media. Following overnight incubation at 30°C, comparable fluorescence was observed for both ENFINIA Plasmid and vendor.

Reporter gene constructs were also tested using a cell-free transcription-translation (TXTL) system. TXTL systems have broad academic and industrial applications for rapid and flexible protein expression, powering foundational biology research, synthetic biology, biomanufacturing, biosensing, biological computing, and more.[4]

We used three reporter genes, mCherry, mNeonGreen (in two codon-optimized versions), and Purple WT, as plasmid templates for myTXTL® Pro Cell-Free Expression. For all three constructs, we observed successful expressions ***(Figure 5)***. It is worth noting that the fluorescence levels of mNeonGreen, a GFP-like protein, are comparable with the GFP-positive control plasmid provided with the kit ***(Figure 5A;*** labeled PC-GFP). Additionally, we observed a slight difference between the two mNeonGreen versions, which might be driven by the different codon optimization strategies ***(Figure 5A)***. We also observed successful expression of the Purple WT reporter, demonstrating the robust functioning of ENFINIA Plasmid DNA across several reporter genes ***(Figure 5B)***.

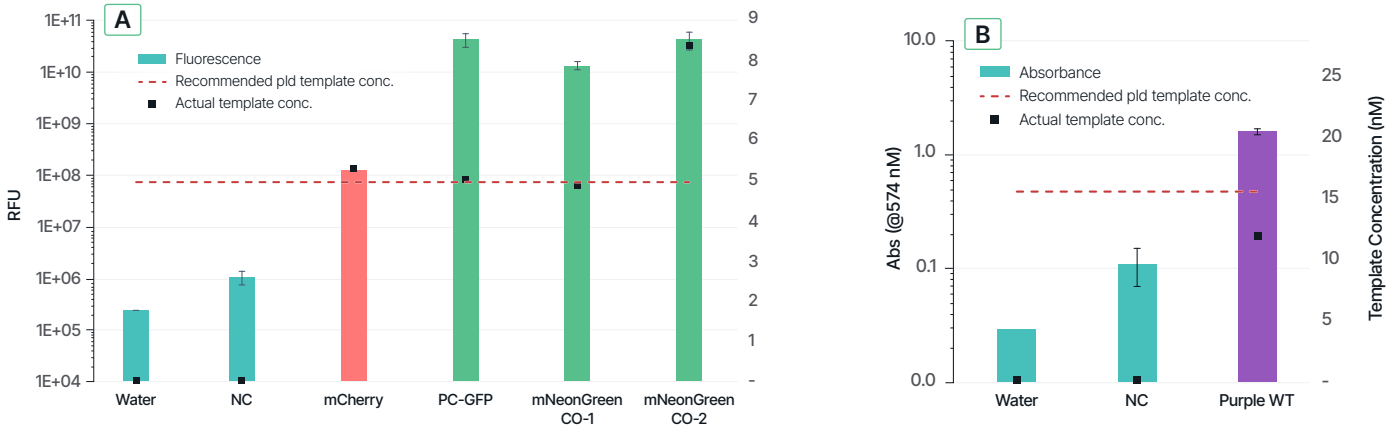

*Figure 5. Expression of reporter genes from ENFINIA plasmid DNA templates in myTXTL® Pro Cell-Free Expression Kit. Reactions were incubated with plasmid DNA templates encoding the mNeonGreen CO-1, mNeonGreen CO-2, mCherry, Purple WT or deGFP (as a positive control, labeled PC-GFP) reporter gene at the indicated concentrations. Water and a myTXTL reaction incubated without a template (NC for Negative Control) were assayed in parallel. Expression was measured following 16 hours of incubation at 27°C. **A)** Expression measured as fluorescence of the reporter gene was quantified using VictoNivo plate reader. Fluorescence measured at Ex/Em 480/530 nm (GFP and mNeonGreen) and 530/600 nm (mCherry). Error bars represent the SEM from 3 technical replicates. **B)** Expression measured as absorbance of the reporter gene Purple WT was quantified using DeNovix DS-11 FX spectrophotometer at 574 nm. Error bars represent the SEM from 3 technical replicates.*

CONCLUSION

The study described here demonstrates that ENFINIA Plasmid DNA can deliver sequence-perfect plasmids with over 92% success across diverse sequence lengths (up to 15 kb) and complexities, circumventing many challenges in traditional molecular cloning and gene synthesis. We also demonstrate that ENFINIA Plasmid DNA is functional, successfully driving the expression of three reporter genes in *E. coli* and in cell-free TXTL systems. Using Elegen for clonal gene synthesis, researchers can receive sequence-perfect, NGS-verified plasmids in as little as 10 days, accelerating their molecular biology and broader research efforts.

References

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