ELEGEN

ENFINIA™ IVT Ready DNA: Cell-Free Linear DNA Templates for mRNA Synthesis

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Abstract

The success of mRNA vaccines during the COVID-19 pandemic highlighted the immense potential of mRNA as a platform for gene and cell therapy. However, key challenges have slowed the adoption of mRNA as a widespread therapeutic tool. Conventional *in vitro* transcription (IVT) workflows that rely on linearized plasmid DNA (pDNA) templates with encoded poly(A) sequences are time-intensive, require lengthy cloning and purification steps, and carry risks of host cell contamination as well as variability.

In contrast, IVT Ready DNA produced using Elegen's proprietary cell-free synthesis platform enables delivery of diverse and complex IVT-ready templates within 10 business days, without compromising mRNA quality or functionality.

This study compares ENFINIA IVT Ready DNA to linearized plasmid DNA as templates for IVT by evaluating template quality, resulting mRNA yield and quality, and the expression level of transcribed eGFP in HeLa mammalian cells. The findings validate use of IVT-ready DNA as a reliable, superior alternative to plasmid-based approaches, overcoming sequence heterogeneity and host cell contamination associated with plasmid-derived DNA templates.

Introduction

Despite rapidly growing interest in mRNA as a therapeutic platform, bottlenecks in DNA supply continue to slow its adoption. Conventional DNA supply relies on cell-based plasmid production for mRNA synthesis, but this approach poses challenges in meeting the critical quality attributes—such as identity, integrity, purity, structure, stability, and function—needed to ensure mRNA safety and efficacy.

mRNA therapeutic workflows include several key steps: DNA template design, template production and purification, IVT reaction for mRNA production, and finally mRNA encapsulation and formulation for delivery and storage. Plasmid-derived templates have been the de facto method of DNA synthesis, and are often selected to reduce costs. However, cell-free DNA manufacturing delivers higher-quality DNA templates with faster turnaround times to enable higher efficiency in the discovery phase and a streamlined path to scale-up for manufacturing, ultimately saving on resources and overall cost.[1]

Plasmid DNA production involves cloning, microbial fermentation, and multiple purification steps, requiring specialized host strains and days to weeks of time at high cost.[2] The process carries risks of downstream contamination from bacterial DNA, endotoxins, and antibiotics, even after purification steps are performed. Maintaining long, homogeneous poly(A) tails throughout cell-based cloning is also challenging due to recombination during bacterial propagation, leading to polydispersity, batch variability, and reduced mRNA yield.[3] Furthermore, circular plasmids must be linearized and purified prior to use in IVT. Recent studies show that extensive purification of linearized templates is essential to minimize dsRNA byproducts, which can trigger innate immune responses and compromise therapeutic efficacy.[4] As manufacturing methods mature, eliminating the reliance on cells is key to ensuring consistent yield, quality, and regulatory compliance in mRNA therapeutics. To address these limitations, Elegen developed cell-free linear ENFINIA IVT Ready DNA as a superior alternative to plasmid-derived templates, validated for integrity, purity, yield, transfection compatibility, and functional protein expression.[5,6]

ENFINIA IVT Ready DNA is produced in a linear format with a wide range of sequence complexity from an entirely cell-free workflow to ensure consistent poly(A) lengths and avoid bacterial contaminants and antibiotic residues. NGS-verified and shipped in as few as 10 business days, this end-to-end cell-free DNA synthesis alternative enables rapid exploration of a larger sequence space of UTR and poly(A) designs for optimal RNA expression.

In this study, eGFP mRNA transcribed from both IVT-ready DNA and linearized plasmid DNA templates was compared by monitoring protein expression via fluorescence over time in HeLa cells.

Study objectives

1. Compare DNA quality of IVT-ready vs. plasmid templates (yield, fidelity, poly(A) tail length).
2. Assess IVT performance and RNA quality from both template types.
3. Evaluate mRNA functionality in mammalian cell-based assays.

Study design

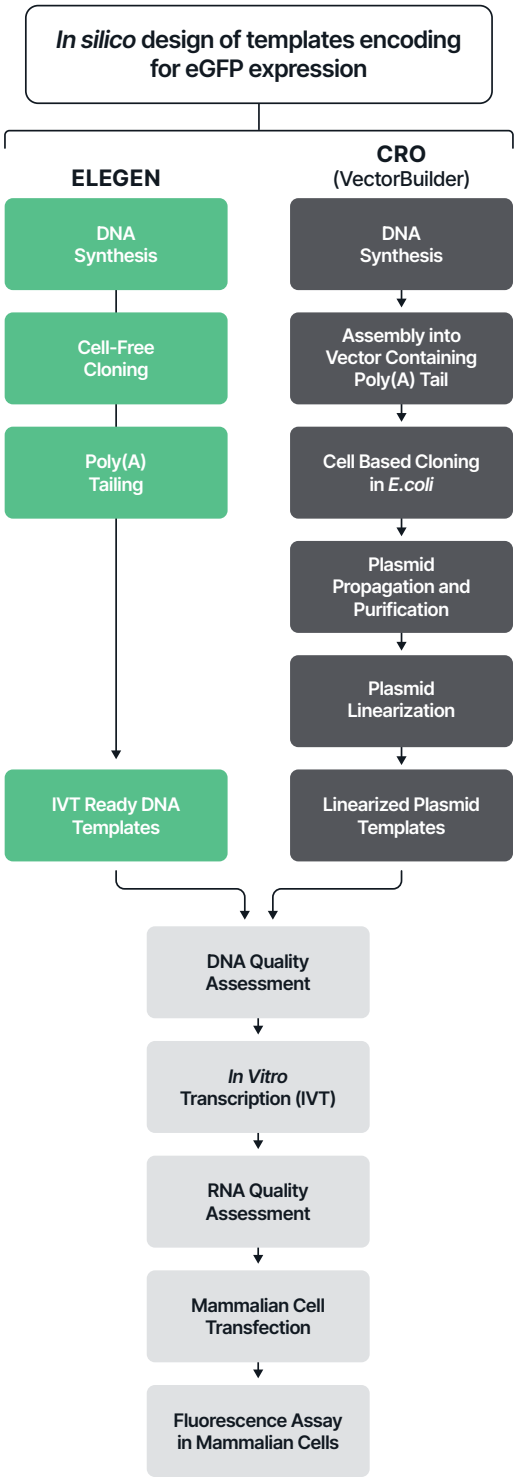

Figure 1. *Study design to compare ENFINIA IVT Ready DNA with linearized plasmid templates using DNA, RNA and cell-based functionality metrics.*

Materials and Methods

DNA template synthesis and characterization

ENFINIA IVT Ready DNA constructs, produced by Elegen, encoded a 903 bp eGFP open reading frame (ORF) that included a T7 promoter, a selected 5′ UTR and 3′ UTR, and a continuous poly(A) sequence of A80 or A130. The templates also included a 5′ process adapter that is upstream of the promoter and therefore not transcribed (***Figure 2***). The constructs were shipped to an external service provider, VectorBuilder Inc. (Chicago, IL), who performed experiments to assess DNA template quality and downstream mRNA quality and expression in comparison to plasmid-derived templates. For comparison, VectorBuilder produced linearized plasmid DNA templates matching the design of each ENFINIA IVT Ready DNA template. VectorBuilder's production process included gene synthesis, transformation, propagation in *Escherichia coli*, and screening by colony PCR followed by plasmid DNA miniprep, purification, and linearization.

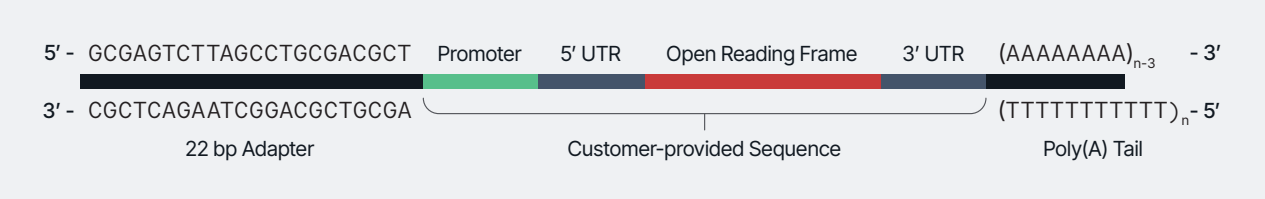

Figure 2. *Schematic diagram of ENFINIA IVT Ready DNA general structure.*

DNA template quality

Genes were synthesized and cloned in a backbone encoding the appropriate poly(A) tail. The final plasmids, pmRVacM-VB-EGFP (A130) and pmRVac-VB-EGFP (A80), were subjected to diagnostic restriction digestion with NruI and ApaLI restriction enzymes, followed by gel-based extraction to determine the relative sizes of the resulting DNA fragments. The sequence of each plasmid was verified by Sanger sequencing. Following linearization with SapI, poly(A) sequence integrity and length were verified by digestion with BtgI followed by PAGE analysis.

The yield of each ENFINIA IVT Ready DNA template was assessed by UV quantification at A260. Chemical purity of each template was assessed by UV quantification at the wavelength ratios A260/A280. The poly(A) sequence integrity and length of each template was verified by digestion with BtgI followed by PAGE analysis.

IVT reactions

VectorBuilder performed IVT reactions following a proprietary protocol. All reactions were performed with 6 µg of template DNA and 100% substitution of uridine with N1-Methylpseudouridine (m1Psi). Cap1 was added by co-transcriptional capping. All IVT reaction products were treated by DNase I to remove untranscribed template DNA. The resulting mRNA from each template was purified by oligo(dT) chromatography, followed by sterile filtration in RNase-free citrate buffer.

Quality of transcribed mRNA

The purified mRNA from each IVT reaction was assessed for

- concentration and chemical purity, as measured by UV spectrophotometry
- sequence accuracy, as measured by cDNA sequencing
- length and integrity, as measured by denaturing agarose gel analysis and by Capillary Gel Electrophoresis (CGE)
- poly(A) tail length, as measured by T1 RNase digestion and urea-PAGE gel electrophoresis
- dsRNA levels, as measured by dot blot analysis

mRNA functionality assessment

The functionality of mRNA produced from each IVT reaction was validated in mammalian cell cultures by quantifying eGFP fluorescence as a measure of protein expression. The day before transfection, HeLa cells were seeded at a density of 2×10^5 cells/well in 12-well plates. A total of five groups were set, including two purified mRNA preps from ENFINIA IVT Ready DNA, two from linearized plasmid templates, and a non-transfected control group *(**Table 1**)*.

Each experimental group was transfected with 1 or 2 µg/well of mRNA in three independent transfections. All untransfected and transfected HeLa cells from each transfection experiment were collected into tubes. At 48 and 72 hours following transfection, each HeLa cell population was analyzed by fluorescence microscopy (Zeiss, Axiover 5), washed with PBS, resuspended, and analyzed by flow cytometry (Beckman, CytoFLEX) to determine the proportion of eGFP-positive cells. A specific exposure time (bright field: 100 ms, 488 nm: 100 ms) was used for each channel during the fluorescence imaging.

Table 1: Experimental setup of transfection

Experimental group		Abbreviation	Dose (µgRNA/ well)	
A	Negative control (cells only)	NC	1	2
B	eGFP mRNA A80 (linearized plasmid)	PLD-eGFP-80	1	2
C	eGFP mRNA A130 (linearized plasmid)	PLD-eGFP-130	1	2
D	eGFP mRNA A80 (ENFINIA IVT Ready DNA)	IRD-eGFP-80	1	2
E	eGFP mRNA A130 (ENFINIA IVT Ready DNA	IRD-eGFP-130	1	2

Note: *All mRNA transcripts were produced using a proprietary IVT protocol, performed by VectorBuilder.*

Results

ENFINIA IVT Ready DNA template quality

Two ENFINIA IVT Ready DNA templates were assessed for yield, chemical purity, sequence fidelity, length, overall presence of poly(A) tailed molecules and polydispersity of poly(A) tails, and the presence of side products. Both templates met the following quality requirements for this study *(**Supplementary Table 3**)*:

- Minimum yield of 40 µg
- Match to reference sequence with no evidence of SNPs or short INDELs
- A260/230 ratio greater than 1.8
- A260/280 ratio greater than 1.8
- Proportion of truncated molecules no greater than 20%
- Proportion of transcribable truncated molecules no greater than 5%
- Proportion of molecules with an intact poly(A) tail greater than 95%
- Poly(A) tail length of either 80 or 130 nt
- No less than 90% of reads per tail are within 15% of the target length

Additionally, the DNA length and integrity of each template was measured on a Bioanalyzer 2100 (Agilent). For each, a single peak was observed at the expected size *(**Figure 3**)*. The poly(A) sequence length and distribution of all templates was assessed using PacBio sequencing. Overall, ENFINIA templates had >90% of reads with a poly(A) sequence of length within 15% of the target length *(**Figure 4**)*.

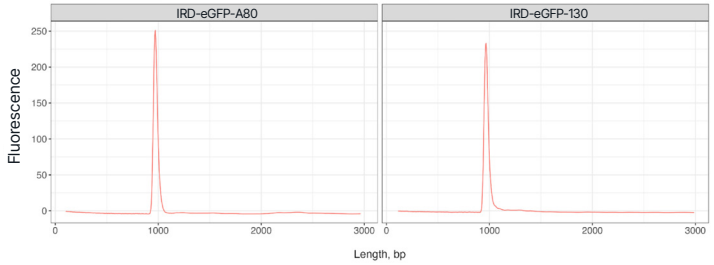

Figure 3. Bioanalyzer traces of ENFINIA IVT Ready DNA templates IRD-eGFP-A80 (left panel) and IRD-eGFP-A130 (right panel).

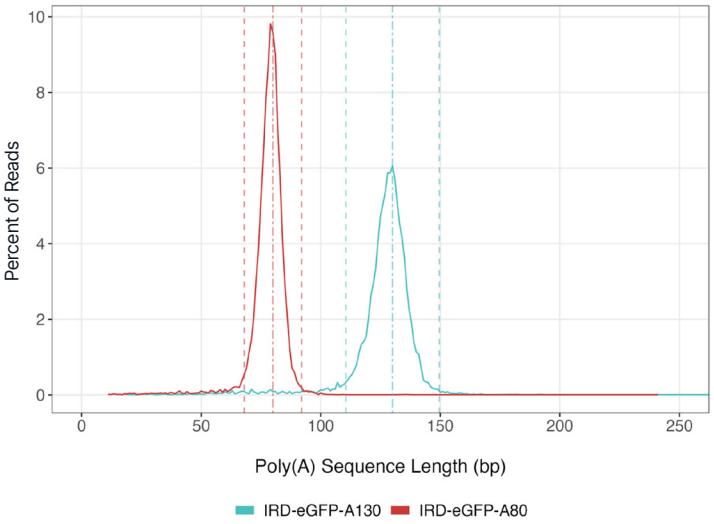

Figure 4. Poly(A) sequence length distribution for each ENFINIA IVT Ready DNA template as determined by long-read PacBio sequencing. The centering and spread of the distribution are within 15% of the target length.

Plasmid-based linearized templates

Each plasmid was evaluated for the correct size and sequence identity by restriction digest and Sanger sequencing, respectively. Both plasmids were at the expected size with 100% alignment to required sequence (***Supplementary Figures 9 & 10***). Poly(A) tail integrity was assessed by PAGE analysis following digestion using BtgI (New England Biolabs). For each plasmid-derived template, a single band was observed at the expected size (results not shown).

Characterization of *in vitro* transcribed RNA

RNA YIELD

Prior to performing IVT reactions, the linearization of the plasmid-derived templates and IVT-ready templates was quantified by gel electrophoresis (1% agarose). All IVT reactions used 6 µg of each DNA template. Overall RNA yields before (primary) and after dT bead purification (final) were comparable ***(Figure 5)***. The slightly lower yield of IVT-ready eGFP A80 and slightly higher yield of IVT-ready eGFP A130 are insignificant and likely the result of experimental variability.

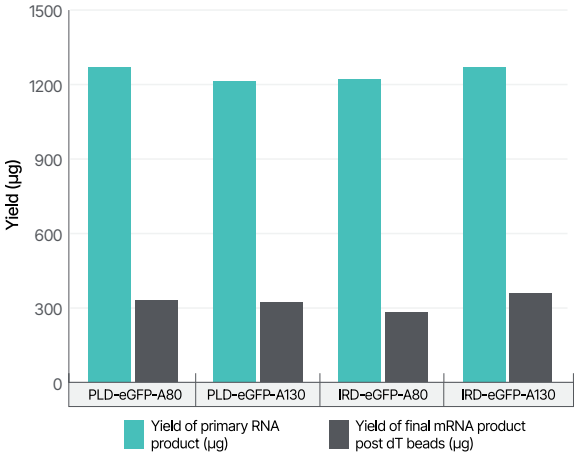

Figure 5: *RNA yield following IVT using 6 µg of each DNA template.*

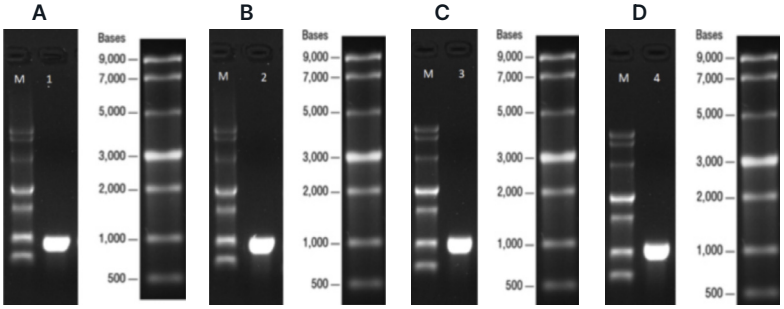

Figure 6: *Size of the mRNA transcript generated from each template as measured by gel electrophoresis. (A) PLD-eGFP-A80, (B) PLD-eGFP-A130 (C)IRD-eGFP-A80 and (D) IRD-eGFP-A130.*

The integrity of the IVT mRNA was visualized by gel electrophoresis and capillary electrophoresis. A single band at the approximate size of 1000 nt was observed by gel electrophoresis for all templates, as expected ***(Figure 6)***. A single peak at the expected retention time was observed by capillary electrophoresis for all templates ***(Figure 7)***.

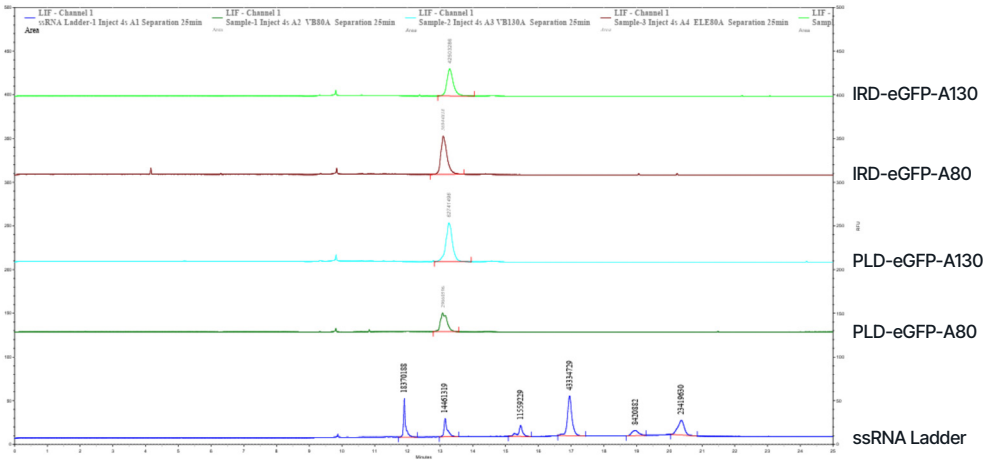

Figure 7: *Size of the mRNA transcript generated by each template as measured by capillary gel electrophoresis.*

mRNA produced from each IVT reaction was digested with RNase T1 and analyzed by Urea-PAGE to verify poly(A) tail length. The expected poly(A) tail length was observed for all templates. Additionally, the fidelity of the mRNA produced in each reaction was measured by Sanger sequencing following reverse transcription and PCR amplification with multiple primers. Each mRNA aligned well to the corresponding reference sequence.

A summary of the characterization of the IVT RNA products is listed in ***Table 3***. Analysis included all the parameters that are critical for optimum performance, including RNA chemical purity, RNA integrity and fidelity, evaluation of dsRNA levels, and mRNA poly(A) tail length and polydispersity. All templates passed acceptance criteria, demonstrating comparable performance.

Table 2: Summary of the results from the analysis of mRNA produced via IVT using each DNA template.

Criteria	Specifications	Plasmid-derived eGFP-A80 VB	Plasmid-derived eGFP-A130 VB	IVT-ready eGFP-A80 Elegen	IVT-ready eGFP-A130 Elegen
RNA Concentration	>500 ng/µL	930.7	728.2	620.9	877.5
Purity[a] A260/280	~2.0	1.90	1.94	1.88	1.91
RNA Length	A sharp band matching the expected size	Verified	Verified	Verified	Verified
RNA Length (CGE)	A peak matching the expected location	Verified	Verified	Verified	Verified
RNA Integrity[b]	≥ 80%	Verified	Verified	Verified	Verified
RNA Sequence Accuracy[c]	Match the reference sequence	Verified	Verified	Verified	Verified
Residual dsRNA[d]	≤30 ng dsRNA/µg RNA (3%)	Verified	Verified	Verified	Verified
Poly(A) length[e]	≥ 90% of theoretical length	Verified	Verified	Verified	Verified

(a) m1Ψ or 5-moU modified RNA with a high ratio of UTP may have a low A260/A280 value.
(b) The RNA integrity was assessed using denaturing agarose gel electrophoresis.
(c) The RNA was reverse transcribed into cDNA, which was then amplified by PCR and subjected to Sanger sequencing.
(d) The sample was spotted onto a nylon membrane, and residual double-stranded RNA (dsRNA) was detected by antibody probing and subsequent imaging.
(e) All mRNA containing a poly(A) sequence was digested with RNase T1 and analyzed by Urea-PAGE to confirm the length of the poly(A) tail.

mRNA FUNCTIONALITY TESTING

The functionality of mRNA generated from each IVT reaction was assessed by measuring the expression of eGFP, measured by fluorescence, in mammalian cells. Expression levels are affected by sequence quality, sequence accuracy, dose accuracy, and transfection efficiency. Fluorescence measured by flow cytometry (***Figure 8***) showed comparable performance across template types.

Flow cytometry analysis confirmed similar cell counts for the negative control and all treatment groups following a 1 µg mRNA dose, regardless of mRNA source. In contrast, a 1.5 - 2x reduced cell count was observed following a 2 µg dose from all mRNA sources, suggesting a negative impact of higher dosing on cell viability. This phenomenon was more apparent with samples analyzed at 48 hours (***Figure 8, Panel A***). Despite the apparent decrease in cell viability following a 2 µg dose, mean fluorescence intensity values (***Figure 8, Panel C***) were similarly comparable across mRNA types.

Under these conditions, poly(A) tail length (80 nt vs. 130 nt) had no detectable impact on cell fluorescence in any treatment group. Indeed, linear regression modelling of the flow cytometry intensity data was unable to detect a statistically significant association of eGFP fluorescence with template type, poly(A) tail length, amount of mRNA transfected, or hours post-transfection. Collectively, these results indicate that IVT- ready DNA templates perform equivalently to linearized plasmid-derived templates in terms of mRNA functionality.

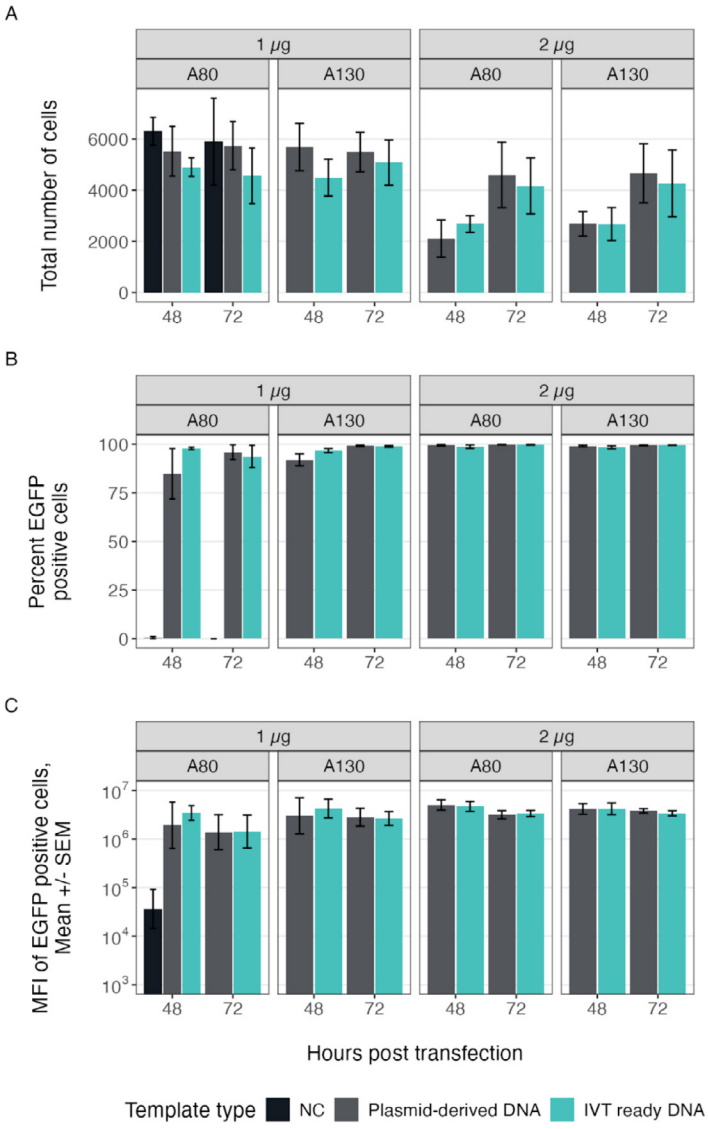

Figure 8: Flow cytometry results of HeLa cells transfected with mRNA product of IVT reactions performed with Elegen IVT Ready DNA templates and plasmid-derived templates.

Results are an average of three independent replicates (mean +/- SEM). Statistical data were derived from flow cytometry detection of eGFP (channel: B525) at 48 h and 72 h post-transfection.

Panel A: total count of cells Panel B: percent of eGFP positive fluorescent cells Panel C: MFI, mean fluorescence intensity of the positive cells.

Discussion

Traditional plasmid-based IVT template generation is slow, labor-intensive, and prone to sequence variability. Cell-free DNA synthesis now enables rapid, high-fidelity production of customized templates, cutting time and cost for mRNA design and optimization. This innovation accelerates development of vaccines, protein and antibody therapies, cell therapies, and genome editing applications.[7]

This study shows that Elegen's ENFINIA IVT Ready DNA is a robust, efficient alternative to linearized plasmid templates. Across all tested parameters—DNA quality, mRNA yield, integrity, purity, and in-cell functionality (eGFP expression in HeLa cells)—IVT-ready linear DNA matched plasmid-derived performance.

Together with prior results[8] for the expression of mCherry and fLuc constructs, the data suggests that ENFINIA IVT Ready DNA can overcome plasmid bottlenecks while maintaining full mRNA functionality. Its high-fidelity, cell-free templates with consistent poly(A) tails enable faster validation and optimization of mRNA therapeutics.

References

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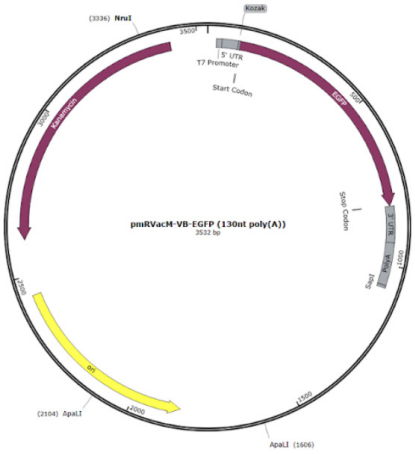
Supplementary

Table 3: Assessment of the quality of all DNA templates tested.

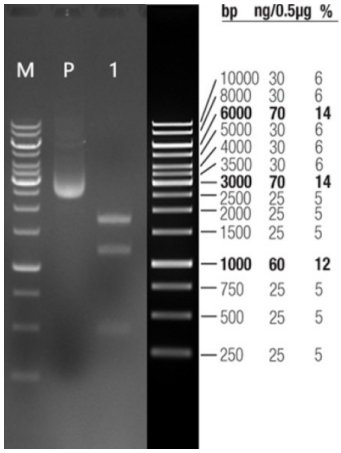
Attribute	Requirement	IVT-ready Elegen		Plasmid VectorBuilder	
		eGFP A80	**eGFP A130**	**eGFP A80**	**eGFP A130**
Yield (µg)	≥40	Verified	Verified	Verified	Verified
Chemical Purity (A260/A230, A260/A280)	>1.8	1.88, 2.02	1.91, 2.02	1.9, 2.06	1.95, 2.03
Sequence Fidelity	No SNPs or small INDELs, NGS-verified	Verified	Verified	Verified By Sanger Sequencing	Verified By Sanger Sequencing
% of Truncated DNA	≤20%	Verified	Verified	Not tested	Not tested
% of Transcribable Truncated DNA	≤5%	Verified	Verified	Not tested	Not tested
Fraction Tailed	≥95%	Verified	Verified	Not tested	Not tested
PolyA Tail Length	Continuous: A80, A130	80	130	80	130
Tail Polydispersity (% of reads with actual poly(A) tail length within 15% of target length, determined by PacBio sequencing, see also Fig. 4)	>90%	94.6%	93.1%	Not tested	Not tested

A: Diagnostic Restriction Digest Analysis



Restriction Enzyme Analysis
NruI+ApaLI (498,1232,1802)

B: Sequence Identity Determined by Sanger Sequencing.



Agarose Gel Picture
Lane: 1

Overview of Sequence Alignment

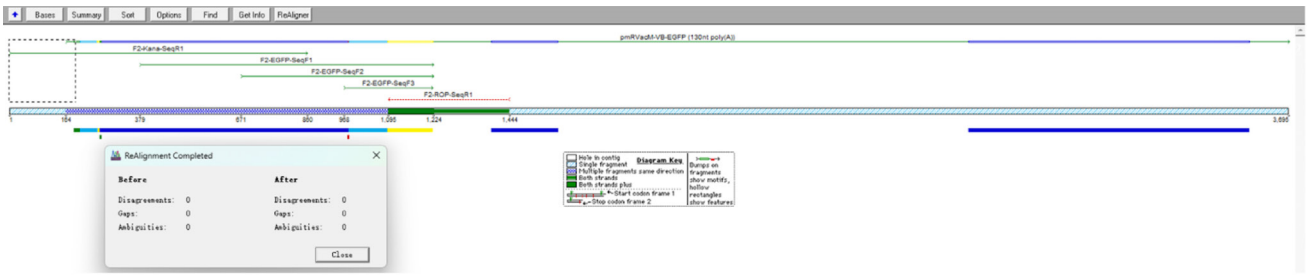
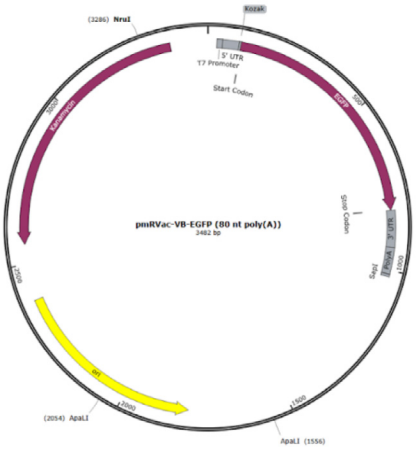


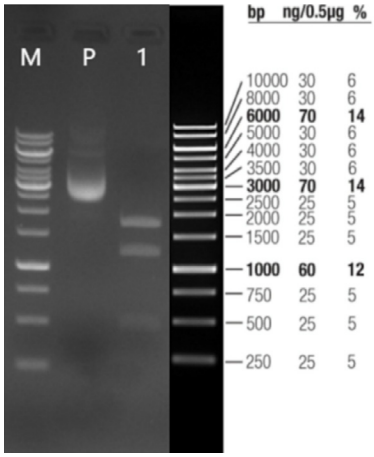
Figure 9: PLD-eGFP-130 quality analyses.

A: Diagnostic Restriction Digest Analysis



Restriction Enzyme Analysis
NruI+ApaLI (498,1232,1752)

B: Sequence Identity Determined by Sanger Sequencing.



Agarose Gel Picture
Lane: 1

Overview of Sequence Alignment

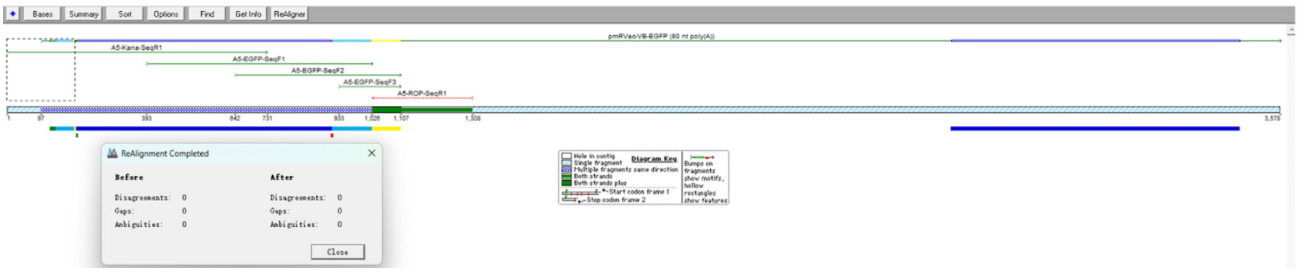


Figure 10: PLD-eGFP-130 quality analyses.