ENFINIA™ IVT Ready DNA is a High-Performance Template for *in vitro* Transcription

Nicholas McGlincy, Ankur Sarkar, Nicholas Reed,
Brooks Bond-Watts, Michael Cariaso, Brittany Enzmann,
Nidhi Gupta, Galit Meshulam-Simon, Sukhvinder Kaur

Abstract

Messenger RNA (mRNA) is a versatile therapeutic platform with applications in infectious disease, cancer, and genetic disorders. Its programmable nature enabled the rapid development of COVID-19 vaccines, but challenges in production—such as speed, fidelity, and batch consistency—remain barriers to broader clinical use. *In vitro* transcription (IVT) using T7 RNA polymerase is the standard method for mRNA synthesis. However, plasmid-based DNA templates suffer from poly(A) tail instability and bacterial impurities.

To overcome these limitations, we developed a cell-free DNA synthesis platform that rapidly produces ENFINIA™ IVT Ready DNA, NGS-verified linear DNA templates with defined poly(A) tails of 70 - 130 bp. Here we compare ENFINIA IVT Ready DNA encoding fLuc and mCherry to conventional plasmids in IVT reactions and evaluate the quality and functionality of the resulting mRNA.

Though dependent on the kit used, IVT-ready templates demonstrate as much as a 1.5-fold higher RNA yield relative to plasmid templates from an equivalent input mass. The resulting mRNA showed improved or equivalent integrity, purity, and poly(A) tail quality. *In vitro* functional tests demonstrate fLuc expression comparable to plasmid-derived mRNA. Results support the use of ENFINIA IVT Ready DNA as a high-quality, reliable, fast, and flexible alternative to plasmid-derived templates to accelerate mRNA therapeutic development.

Introduction

Messenger RNA (mRNA) has been studied as a therapeutic tool since the 1990s.[1] These groundbreaking studies enabled the unprecedented rapid development of the COVID-19 mRNA vaccines. The COVID-19 pandemic highlighted the numerous advantages that mRNA vaccines have over protein-based vaccines during their development, including the ability to rapidly reconfigure their sequence design. This attribute can accelerate development timelines and has enabled mRNA vaccines to rapidly adapt to emerging SARS-CoV-2 variants.[2,3] mRNA's therapeutic potential extends far beyond infectious disease prevention. As a programmable and transient genetic platform, its use is expanding into many therapeutic areas, including treatments for cancer, autoimmune disease, and rare genetic disorders.[4]

Key bottlenecks in mRNA production include long turnaround times for plasmid construction, instability of poly(A) sequences, and batch-to-batch variability during bacterial propagation. High purity and batch-to-batch consistency are critical factors for clinical translation. Ensuring rigorous quality control at scale requires precise characterization of key mRNA attributes—identity, integrity, purity, structural features, stability, and functional activity— critical to the development of safe and effective mRNA-based therapeutics.[5,6]

mRNA is commonly prepared by *in vitro* transcription (IVT), in which phage RNA polymerases (RNAP) such as T7 RNAP transcribe DNA templates into RNA. DNA templates must encode a promoter for the RNAP, followed by all the functional sequence elements of an mRNA: sequences to direct capping, the 5′ and 3′ untranslated regions (UTR), the ORF of interest, and an encoded poly(A) sequence that will become the mRNA's poly(A) tail.

IVT templates are typically derived from a linearized plasmid. However, plasmid-based production presents challenges including poly(A) recombination, bacterial DNA carryover, and endotoxin contamination. Generation and maintenance of plasmids with consistent lengths of poly(A) sequences is a challenging task. Longer poly(A) sequences frequently recombine during bacterial growth, resulting in a shortening of the average poly(A) length and an increase in intermolecular heterogeneity. Plasmid propagation involves growth of bacterial cultures, bacterial harvesting and lysis, and multiple filtration steps.[7] Typically, special *Escherichia coli* strains engineered for the successful propagation and maintenance of long poly(A) sequences will be required. The inherent complexity of these processes, combined with the additional purification steps required after plasmid linearization, often results in significant batch effects. Another frequent issue requiring purification of RNA produced from IVT is the generation of IVT byproducts. These impurities can adversely affect the efficacy of mRNA therapy.[8]

We generated NGS-verified linear templates using cell-free DNA synthesis, thus eliminating plasmid propagation and its associated risks of poly(A) sequence instability, endotoxin and genomic DNA contaminations, and antibiotic residues. This approach enables rapid, low-cost screening of diverse UTRs and poly(A) tail lengths across a broader and more complex sequence space with greater speed and lower cost.

In this paper, we present data comparing the performance of linear IVT-ready DNA templates with conventional linearized plasmid templates for IVT-mediated mRNA production, which demonstrates the robustness and overall effectiveness of our cell-free DNA manufacturing process.

Study objectives

1. Compare DNA quality attributes (yield, sequence fidelity, poly(A) tail length distribution) between IVT-ready DNA and plasmid-derived templates.
2. Compare the performance of IVT-ready DNA templates with plasmid-derived templates in IVT reactions. Understand the effect of template concentration on mRNA yield for both templates and evaluate the quality of the resulting mRNA.
3. Compare the *in vitro* functionality of the mRNA transcribed from IVT-ready DNA templates with that from plasmid-derived templates.

Study design

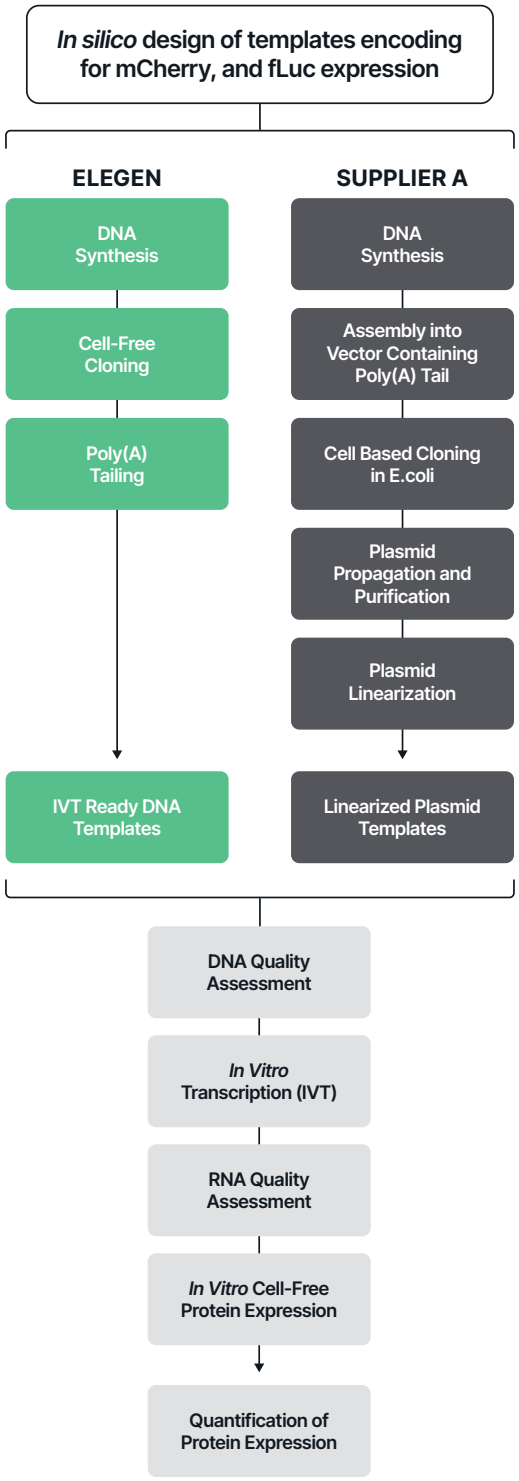


Figure 1. *Outline of study performed to compare IVT-ready and conventional linearized plasmid templates by assessing DNA quality, mRNA quality, and protein expression.*

Materials and Methods

DNA template synthesis and characterization

Four templates encoding monomeric Cherry Fluorescent Protein (mCherry) or Firefly Luciferase (fLuc) with continuous A130 or A90 poly(A) sequences were synthesized by Elegen as ENFINIA IVT Ready DNA and a market-leading gene synthesis supplier (Supplier A) as a plasmid-derived template, resulting in a total of 8 templates for the study. Each template includes a T7 promoter and a selected 5′ UTR and 3′ UTR, both derived from *Homo sapiens* hemoglobin subunit alpha 1 and associated with high mRNA stability and translation efficiency.[9] ENFINIA IVT Ready DNA also includes a 5′ process adapter that is upstream of the promoter and therefore not transcribed ***(Figure 2)***. Supplier A's plasmid production process included gene synthesis, cloning, mini-prep scale propagation, and purification.

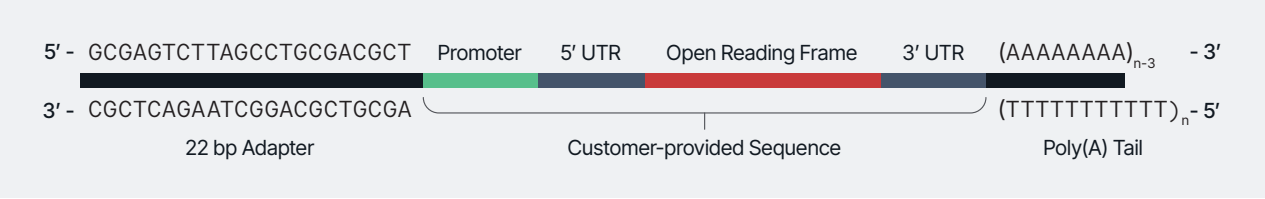


Figure 2. Schematic of a generic linear IVT-ready DNA template.

DNA template QC

Yield of each IVT-ready DNA template was evaluated using the Qubit 1X dsDNA High Sensitivity (HS) Assay Kit (Invitrogen). Chemical purity of each IVT-ready DNA template was measured by UV wavelength ratios A260/A280 & A260/A230 using a DeNovix DS-11 FX spectrophotometer. Each template was sequenced on an Oxford Nanopore Technologies (ONT) MinION using their Native Barcoding Kit 96 V14 library preparation. The sequencing results were analyzed with Elegen's pipeline and used to evaluate sequence fidelity, potential DNA side products, and the fraction of molecules with a poly(A) sequence. The yield, purity, and confirmation of the poly(A) sequence length (Sanger sequencing) were provided for each plasmid template by Supplier A. The length and poly(A) polydispersity of the plasmid and IVT-ready DNA templates were determined by PacBio sequencing using the HiFi plex prep kit on the Vega platform.

IVT reactions

Plasmids were linearized by digestion with BspQI, followed by DNA purification with KAPA Pure Beads (Roche). Linearized plasmids were then quantified using the Qubit 1X dsDNA HS Assay Kit (Invitrogen) and tested in parallel with IVT-ready DNA templates. IVT reactions were performed with the MEGAscript T7 Transcription Kit (Thermo Fisher), according to the manufacturer's instructions. Unless otherwise stated, an equal mass of DNA template was used in all template-type comparisons. After 2 hours at 37°C, IVT reactions were stopped by the addition of EDTA to a final concentration of 50 mM. RNA was then purified from the IVT reaction using an RNeasy mini column according to the manufacturer's instructions, with the recommended on-column RNase-free DNASE I digestion to destroy any residual DNA template. RNA was eluted from the column by two washes with the same 50 µL aliquot of nuclease-free water according to the manufacturer's instructions.

mRNA CHARACTERIZATION

Yield, purity, integrity

RNA yield and chemical purity were measured by UV absorbance and by wavelength ratios A260/A280 & A260/A230 using a DeNovix DS-11 FX spectrophotometer. The production of a single RNA species of the expected size was assayed by running RNA samples on the Agilent 2100 BioAnalyzer system using an RNA 6000 Pico kit according to the manufacturer's instructions. Before analysis, RNA samples were diluted in 0.1 mM EDTA to a concentration within the linear range of the RNA Pico kit (500-5000 pg/µL). Then they were heated to 70°C for 2 minutes and snap-cooled on ice.

Sequence fidelity and poly(A) tails characterization

mRNA sequence fidelity was determined by PacBio sequencing using the Iso-Seq Express 2.0 kit and a custom analysis pipeline.

mRNA poly(A) tail length distribution was characterized by the T1-LCMS method.[10] For each sample analyzed, approximately 120 pmols of *in vitro* transcribed mRNA was subjected to digestion with RNase T1 at a concentration of 16.7 U/pmol mRNA. RNase T1 cuts RNA after G residues, so the major digestion product of an mRNA is expected to be the poly(A) tail. Poly(A) sequences can then be identified using LC-MS. Samples were analyzed at Novatia LLC using a Thermo Scientific Orbitrap Exploris MX coupled with a Thermo Scientific Vanquish HPLC. The analytical column used was a Clarity Oligo-xt column (2.1 x 50 mm, 2.6 µm) from Phenomenex. The mobile phase consisted of water with hexafluoro-2-propanol 1% and 0.1% N, N-Diisopropylethylamine (A) and 35% water 65% ACN with hexafluoro-2-propanol 0.75% and 0.375% N, N-Disopropylethylamine (B). The system was operated at a flow rate of 400 µL/min. The column temperature was 60°C. The gradient condition was as follows: 2%B hold 0-1 min, 6-25%B over 19 min. MS acquisition was done in negative ion mode with a data acquisition range of 500-2000 m/z. Deconvolution was done using Novatia's ProMass software for Excalibur V5.2.

RNA FUNCTIONALITY

Cell-free protein expression (CFPE)

CFPE was performed using Thermo Scientific's 1-Step Human Coupled IVT Kit. For each fLuc encoding template, capped mRNA was produced using the HiScribe CleanCap AG kit from New England Biolabs. Following the manufacturer's protocol, each CFPE reaction was initiated with 2.25 µg of RNA and incubated at 30°C for 5 hours. We utilized 2.25 µg of CleanCap Firefly Luciferase mRNA (TriLink Biotechnologies) as a positive control.

Measuring luciferase activity

One tenth of the CFPE reaction volume was added to 50 µL of the working solution constructed from Thermo Scientific's Pierce Firefly Luciferase Glow Assay Kit. After 10 minutes of room-temperature shaking at 300 rpm, the luminescence was measured using a VICTOR Nivo multimodal plate reader (PerkinElmer).

Results

SPEED OF DNA TEMPLATE PRODUCTION

Template synthesis time

All IVT-ready DNA templates were made with the required regulatory elements and poly(A) sequence lengths. All constructs were of high sequence complexity. In both mCherry and fLuc, the 3′ UTR forms a pseudopolymer, which together with the coding sequence results in a high local GC content (70 - 76%). In addition, the fLuc ORF contains an 18 bp repeat sequence. The average turnaround time (TAT) was 12 business days.

Plasmids from Supplier A containing an A90 poly(A) sequence were shipped at 15 and 27 business days from order. A plasmid with the mCherry gene and an A130 poly(A) sequence arrived in 40 business days, and a plasmid with the fLuc gene and an A123 poly(A) sequence arrived in 52 business days. Supplier A was unable to produce a plasmid containing the fLuc gene and an A130 poly(A) sequence for the study.

Table 1. Comparison of the turnaround times(*) for ENFINIA IVT Ready DNA templates from Elegen and linearized plasmid templates from Supplier A.

Supplier	Gene	ORF Size	Template Type	Poly(A) Length (bp)	TAT (business days*)
Elegen	mCherry	708	Linear	A90	12
				A130	12
	fLuc	1650	Linear	A90	12
				A130	12
Supplier A	mCherry	708	Plasmid	A90	15
				A130	40
	fLuc	1650	Plasmid	A90	27
				A123**	52

**TAT (turnaround time) is based on business days from order submission to shipment.*

*** Supplier A was unable to deliver this gene with the A130 sequence.*

Comparison of DNA template quality

All DNA templates were assessed for yield, chemical purity, sequence fidelity, length, and polydispersity of poly(A) tails. Additionally, all IVT-ready templates were evaluated for the overall presence of poly(A) tailed molecules and the presence of side products. The quality requirements for this study were as follows:

- Minimum yield of 10 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 90 or 130 nt
- No less than 90% of reads per tail are within 15% of the target length

As shown in Supplementary ***Tables 2 and 3***, each IVT-ready template met all quality requirements for the study. The linearized plasmid templates from Supplier A met the requirements for yield, chemical purity, and sequence fidelity. All plasmid templates for mCherry and fLuc show a higher degree of polydispersity, with less than 90% of reads per tail within 15% of the target sequence length. The plasmid template for fLuc with an A130 tail was measured to be 123 bp by Sanger sequencing.

We performed a visual inspection of the IVT-ready DNA templates and an equivalent mass of the linearized plasmid template on an Invitrogen E-Gel EX 1% Agarose. Results, shown in ***Figure 3***, demonstrate a single band that closely matches the expected size.

We measured the distribution of poly(A) sequence lengths (polydispersity) for each template using long-read PacBio sequencing. Results for the IVT-ready DNA templates and the linearized plasmid templates demonstrated comparable or better poly(A) sequence length distributions. A higher frequency of molecules with shorter poly(A) sequences was observed from linearized plasmids, particularly in the mCherry A130 sample ***(Figure 4)***. Overall, for IVT-ready DNA templates, >90% reads were within 15% of tail length target, while for plasmids only 63 - 82% reads were within 15% of the target tail length.

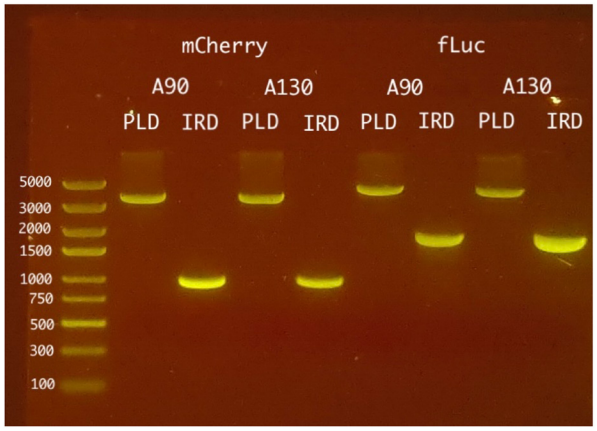


Figure 3. Agarose gel electrophoresis of linearized template DNA. 100 ng of each template was run on a 1% agarose gel. Sizes were estimated based on the migration of E-Gel 1 Kb Plus Express DNA Ladder. Note: the expected size of plasmids is larger than the IVT-ready DNA template, as it includes the vector backbone.

PLD is Plasmid-derived DNA, IRD is IVT-ready DNA. Expected product sizes in bp from left to right are: 3504, 925, 3604, 1025, 4506, 1927, 4546, 1967.

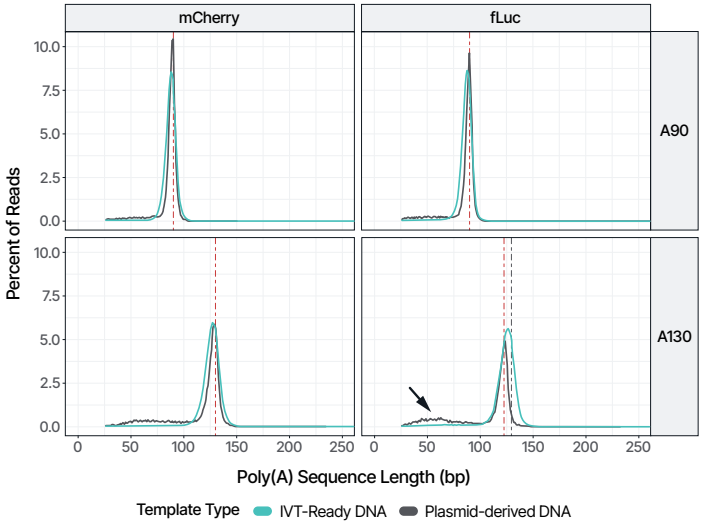


Figure 4. Poly(A) sequence length distribution of IVT-ready DNA and linearized plasmid-derived DNA templates as determined by long-read PacBio sequencing. The centering of the distribution around the expected size of the poly(A) sequence (dashed line) and the degree of polydispersity of the poly(A) sequence are comparable between the two template types and within 15% of the target. A higher frequency of molecules with shorter poly(A) sequences was observed from linearized plasmid-derived DNA templates, especially in the A130 samples (arrow). Results for the fLuc plasmid-derived template with the designed A130 poly(A) tail confirm the Sanger sequencing results provided by Supplier A. The actual size of the poly(A) sequence is less than 130 nucleotides.

CHARACTERIZATION OF *IN VITRO* TRANSCRIBED RNA

RNA yield

Parameters that affect RNA yield are numerous and include the ORF length, template concentration, the type of promoter, the rNTPs concentration, and the kit used. The observed RNA yields were within the anticipated range for a two-hour transcription employing the MEGAScript T7 transcription kit (ThermoFisher).[11] Compared to the equivalent mass of linearized plasmid, IVT-ready DNA templates generated ~1.5-fold higher RNA yields. A higher yield was consistently observed across the two genes and two poly(A) tail lengths tested ***(Figure 5)***. This advantage is likely due to the higher molarity of the ENFINIA template, which includes only the gene of interest and the necessary elements for transcription.

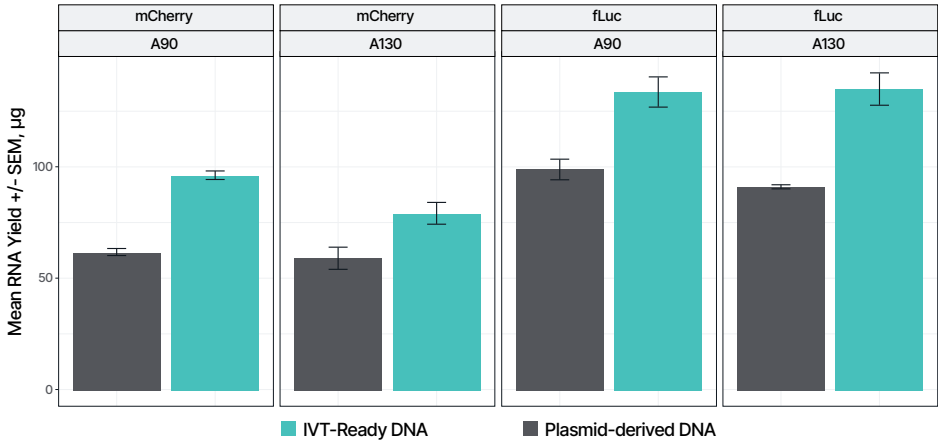
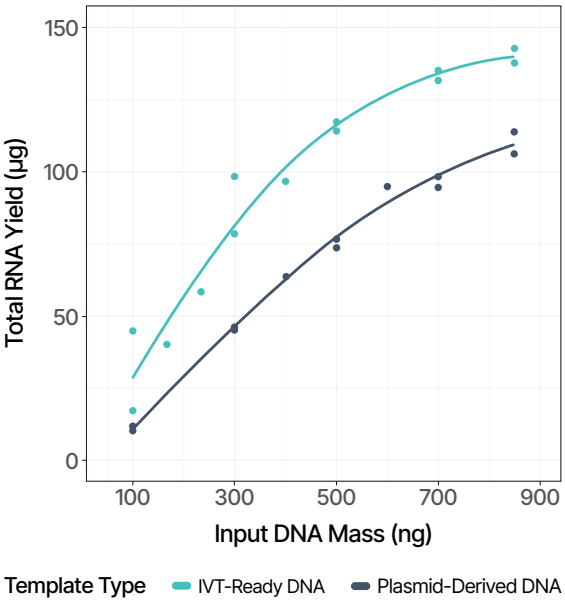


Figure 5. RNA yield as measured by UV absorbance from IVT reactions using the IVT-ready DNA or plasmid-derived templates. IVT-ready DNA templates yield higher RNA amounts compared to linearized plasmid templates when using the same input mass. IVT-ready DNA and plasmid template input mass: 500 ng. Bars represent mean ± SEM, n=3-6.



Impact of template mass on RNA yield of IVT reactions

Using both template types encoding mCherry with a 90 bp poly(A) sequence and the T7 MegaScript kit, we examined the impact of titrating template mass on RNA yield. Similar to the results illustrated in Figure 5, we observed an approximately 1.5-fold increase in RNA yield from IVT-ready DNA templates as compared to linearized plasmid templates across a template mass of 100-900 ng (see ***Figure 6***).

It is reasonable to attribute this outcome to the higher molarity of the IVT-ready DNA templates, which contain only the gene of interest along with the essential transcription elements.

Figure 6. IVT reactions performed with IVT-ready DNA yield more RNA than those with an equivalent mass of linearized plasmid template across all tested concentrations.

Templates used: mCherry with 90 bp poly(A) sequence. IVT-ready DNA length: 0.93 kb. Plasmid-derived DNA length: 3.6 kb. Each point represents one IVT reaction. For each template type, the line of best fit was calculated by LOESS regression.

RNA chemical purity

The chemical purity of RNA isolated from IVT reactions was measured by the well-characterized UV spectrophotometric ratios A260/A280 and A260/A230. Overall, results are comparable between RNA generated from IVT-ready DNA and linearized plasmid templates (see Supplementary ***Table 4***).

Generally, the A260/A280 ratios observed were slightly higher than the desired range (1.8 - 2) but showed no difference between IVT-ready DNA and linearized plasmid templates. A possible explanation for this might be the Qiagen RNeasy column-based purification method used for IVT reaction clean-up. Purification of RNA from IVT reactions is an area of active research, especially of large-scale reactions. It may be that a more involved method would have resulted in higher chemical purity.[12]

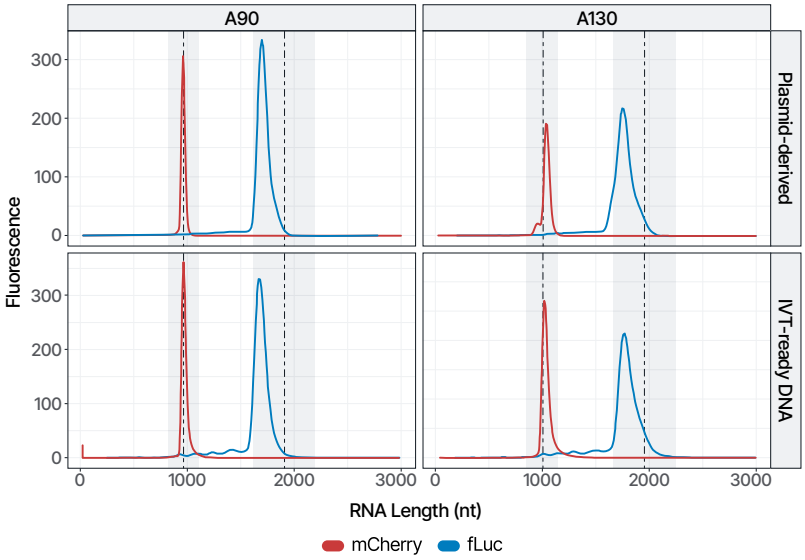


Figure 7. *RNA integrity of representative IVT RNA samples as measured by an Agilent 2100 BioAnalyzer using the RNA 6000 Pico kit. The expected size is indicated by a vertical dashed line, with a shaded box representing ±15 % of the expected size. Vertical facets represent the designed poly(A) sequence length.*

RNA integrity & fidelity

The generation of a single RNA product of the expected size is a critical quality attribute for many *in vivo* applications of the transcribed RNA, especially mRNA therapeutics and vaccines. IVT reactions often generate impurities, including elongated or truncated transcripts, uncapped RNA, and double-stranded RNA (dsRNA). Such impurities should be minimized, as they may impair translational efficiency and trigger unwanted immune responses *in vivo*. In the clinical setting, this will manifest as a reduction in tolerability and therapeutic efficacy. Indeed, the FDA guidelines for characterizing impurities are extensive.[13–15]

The production of a single major RNA species of approximately the expected length was confirmed for all templates (see ***Figure 7***). In all cases, the size of the prominent peaks was within 15% of the expected size. Moreover, high RNA integrity was demonstrated by all prominent peaks, which represented more than 85% of the total area under the curve for each trace.

RNA fidelity, i.e., the expression of the intended RNA sequence, was confirmed by long-read RNA-seq of IVT products using the PacBio Iso-Seq kit. No SNPs or deletions were detected, confirming the high quality of the transcript.

mRNA poly(A) tail length and polydispersity

The mRNA 3′ poly(A) tail is vital for efficient translation and stability *in vivo*.[16] In the eukaryotic cytoplasm, the poly(A) tail promotes translation by recruiting poly(A)-binding protein (PABP). PABP, in turn, interacts with the eIF4F complex, forming closed-loop mRNA and promoting translation initiation. Furthermore, the poly(A) tail protects the mRNA from exonucleolytic degradation, enhancing transcript stability and thereby supporting higher protein expression.[17] The number of A residues is variable, typically ranging from 30 to 160 nt in mammals.[16]

Poly(A) tails can be introduced into IVT RNA either co-transcriptionally, by encoding the tail in the DNA template, or post-transcriptionally, by treatment with *E. coli* Poly(A) Polymerase. Enzymatically added tails exhibit broader length distributions than plasmid-encoded tails, but the latter are unstable to maintain, especially with long poly(dA:dT) sequences (>80 bp).[9] Accurate measurement of poly(A) tail length is essential for linking tail properties to mRNA stability and protein expression. Among the several common methods, such as sequencing, electrophoretic, and chromatographic methods, mass spectrometry (MS) enables direct, label-free measurement at single-nucleotide resolution. It can simultaneously quantify multiple oligonucleotides, making it the method of choice for this study.[9]

To measure poly(A) tail length by LC-MS, mRNA was digested with RNase T1, which cleaves after G residues, yielding the poly(A) tail as the major product. Tail length distribution was then determined from the size distribution of these digestion products (see Materials & Methods).

As shown in ***Figure 8***, the digested poly(A) tails of RNA generated from IVT-ready DNA and linearized plasmid templates exhibit comparable poly(A) tail lengths and polydispersity. Moreover, we can observe the expected 123 nt poly(A) tail on RNA generated from the plasmid template for fLuc, which was designed with a 130 bp poly(A) sequence.

Consistent with previous publications, the tail for each template presents as a distribution rather than a single mass.[10,18] The distribution is biased towards higher mass products rather than the encoded poly(dT:dA) length, presumably as a result of the well-described transcription slippage or arrest of the RNAP.[19]

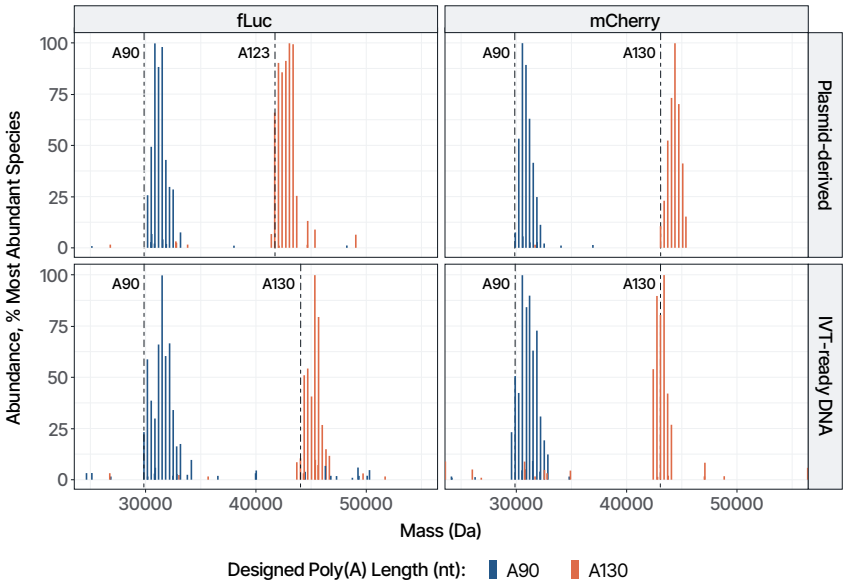


Figure 8. Comparable poly(A) tail lengths and polydispersity observed by LC-MS analysis of RNAse T1 digestion products for fLuc MEGAscript RNA.

In vitro functionality testing

Beyond standard quality assessments, we assessed the functionality of the mRNA produced from each template type. Here, we utilized a cell-free protein expression (CFPE) system to evaluate translation by expressing the fLuc protein from synthesized mRNA, incorporating attributes such as capping and poly(A) tail. To assay this capped mRNA, constructs encoding fLuc were transcribed using NEB's HiScribe CleanCap AG kit. An equal mass of mRNA from each template was then used in a ThermoFisher 1-step human *in vitro* translation kit to generate protein. fLuc protein expression was then assessed by measuring the luciferase activity of the protein extract using the ThermoFisher Pierce Glow Assay. ***Figure 9*** demonstrates comparable luciferase activity across the different template types and poly(A) tail lengths.

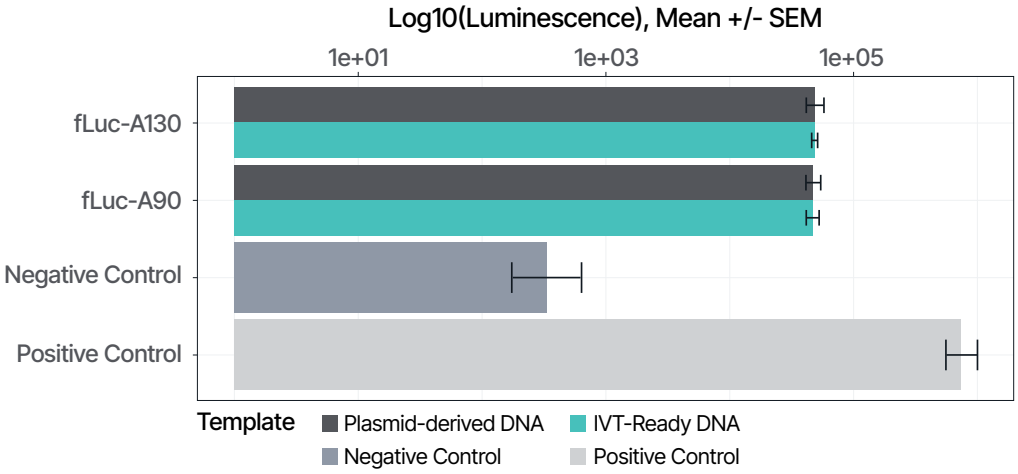


Figure 9. Luminescence of CFPE on RNA generated with HiScribe T7 CleanCap AG kit. Bar length represents the mean +/- SEM of three replicate CFPE reactions, each with mRNA from an independent IVT reaction. The negative control is produced from reactions without mRNA. The positive control consists of CleanCap Firefly Luciferase mRNA (TriLink Biotechnologies).

Discussion

Cell-free DNA synthesis has reduced reliance on bacterial propagation of plasmid DNA, enabling faster turnaround of custom templates for mRNA synthesis. Researchers can now rapidly fine-tune mRNA sequences for improved stability and efficacy, making mRNA production a powerful platform for prophylactic and personalized cancer vaccines, protein replacement and antibody therapies, cell therapies, and genome editing. Cell-free gene synthesis accelerates therapeutic development timelines by enabling rapid synthesis of DNA templates for mRNA design and optimization.[3,20]

In this study, we evaluated the turnaround time, quality of DNA and RNA, and functionality of mRNA produced using Elegen's ENFINIA IVT Ready DNA and conventional plasmid-based templates. Our results show a reduction in turnaround time (TAT) for IVT-ready high-complexity DNA templates compared to plasmid-derived templates, with a TAT of 12 days versus 18-52 days. Additionally, IVT-ready DNA templates required no amplification, linearization, or polyadenylation. This reduced turnaround time facilitates the faster iteration of mRNA construct designs, thereby accelerating therapeutic development.

DNA quality assessments revealed that IVT-ready DNA exhibited quality characteristics equal to or superior to those of linearized plasmid templates. While DNA integrity was largely consistent across formats, linearized plasmid templates were produced with a higher proportion of truncated poly(A) sequences. Additionally, the inability of the selected plasmid supplier to deliver the A130 sequence may be a direct consequence of reduced poly(A) stability in their cell-based cloning process.

The evaluation of desired RNA characteristics demonstrated comparable traits across IVT-ready DNA and linearized plasmid-derived DNA, with overall higher RNA yields for IVT-ready DNA across a range of equal input masses. We relate this outcome to the higher molarity of the IVT-ready DNA templates, which contain only the gene of interest along with the essential transcription elements. An additional contributing factor may be genomic DNA contamination in the plasmid-derived template, which can reduce the effective molarity of the reaction. Overall, these results suggest that IVT-ready DNA templates are compatible with a commercially available IVT kit and may yield more RNA per template input mass than linearized plasmid-derived DNA templates.

We demonstrated that IVT-ready DNA templates produced mRNA of equivalent functionality to plasmid-derived DNA templates. CFPE of capped fLuc encoding mRNA revealed equivalent luminescence from both template types and poly(A) tail lengths.

Overall, these findings support use of ENFINIA IVT Ready DNA as an NGS-verified, flexible, and rapid cell-free alternative to linearized plasmid-derived templates, accelerating mRNA discovery and scale-up. Future work will evaluate mRNA performance in cell-based assays.

Supplementary

Table 2. mCherry DNA templates QC

Attribute	Requirement	ENFINIA IVT Ready Elegen		Plasmid Supplier A	
		mCherry A90	mCherry A130	mCherry A90	mCherry A130
Yield (µg)*	≥10	Pass	Pass	Pass	Pass
Chemical purity (A260/A230, A260/A280)	>1.8	2.17, 1.91	2.19, 1.88	2.15, 1.98	2.14, 1.99
Sequence fidelity	no SNPs or small INDELs, NGS-verified	Pass	Pass	Pass	Pass
% of truncated DNA	≤20%	Pass	Pass	Not tested	Not tested
% of transcribable truncated DNA	≤5%	Pass	Pass	Not tested	Not tested
Fraction tailed	≥95%	Pass	Pass	Not tested	Not tested
Poly(A) tail length	Continuous: A90, A130	90	130	90	130
Tail polydispersity**	>90%	94.2%	93.3%	85.0%	72.4%

**IVT-ready DNA yield is measured by Qubit.*

***% of reads with actual poly(A) tail length within 15% of target length, by PacBio sequencing, see also Figure 2.*

Table 3. fLuc DNA templates QC

Attribute	Requirement	ENFINIA IVT Ready Elegen		Plasmid Supplier A	
		fLuc A90	fLuc A130	fLuc A90	fLuc A130
Yield (µg)*	≥10	Pass	Pass	Pass	Pass
Chemical purity (A260/A230, A260/A280)	>1.8	2.14, 1.94	2.12, 1.92	2.12, 2.00	2.14, 2.00
Sequence fidelity	no SNPs or small INDELs, NGS-verified	Pass	Pass	Pass	Pass
% of truncated DNA	≤20%	Pass	Pass	Not tested	Not tested
% of transcribable truncated DNA	≤5%	Pass	Pass	Not tested	Not tested
Fraction tailed	≥95%	Pass	Pass	Not tested	Not tested
Poly(A) tail length	Continuous: A90, A130	90	130	90	123
Tail polydispersity***	>90%	92.9%	91.0%	81.2%	63.1%**

**IVT-ready DNA yield is measured by Qubit.*

***Note: Actual fLuc plasmid tail was A123 as the designed A130 couldn't be built. The value here relates to the designed A130.*

****% of reads with actual poly(A) tail length within 15% of target length, by PacBio sequencing, see also Figure 2.*

Table 4. Chemical purity of IVT RNA product

				A260/A280		A260/A230	
ORF	Poly(A) Length	Template Type	N	Mean	CV (%)	Mean	CV (%)
mCherry	90	Plasmid-derived DNA	6	2.25	2.44	2.02	10.00
mCherry	90	IVT-ready DNA	6	2.25	0.59	2.09	7.88
mCherry	130	Plasmid-derived DNA	6	2.27	0.81	2.26	0.758
mCherry	130	IVT-ready DNA	6	2.29	1.22	2.18	2.89
fLuc	90	Plasmid-derived DNA	3	2.11	1.245	2.01	7.44
fLuc	90	IVT-ready DNA	3	2.15	0.44	2.07	4.43
fLuc	123	Plasmid-derived DNA	3	2.13	1.13	2.11	3.94
fLuc	130	IVT-ready DNA	3	2.18	0.99	2.10	6.14

References

1. Wolff, J. A. et al. **Direct gene transfer into mouse muscle *in vivo*.** Science 247, 1465–1468 (1990).
2. Wu, Y., Zhang, H., Meng, L., Li, F. & Yu, C. **Comparison of immune responses elicited by SARS-CoV-2 mRNA and recombinant protein vaccine candidates**. Front. Immunol. 13, 906457 (2022).
3. Chaudhary, N., Weissman, D. & Whitehead, K. A. **mRNA vaccines for infectious diseases: principles, delivery and clinical translation.** Nat. Rev. Drug Discov. 20, 817–838 (2021).
4. Zhang, G., Tang, T., Chen, Y., Huang, X. & Liang, T. **mRNA vaccines in disease prevention and treatment.** Signal Transduct. Target. Ther. 8, 365 (2023).
5. Zhang, H. et al. **Algorithm for optimized mRNA design improves stability and immunogenicity.** Nature 621, 396–403 (2023).
6. Leppek, K. et al. **Combinatorial optimization of mRNA structure, stability, and translation for RNA-based therapeutics.** Nat. Commun. 13, 1536 (2022).
7. Martínez, J., Lampaya, V., Larraga, A., Magallón, H. & Casabona, D. **Purification of linearized template plasmid DNA decreases double-stranded RNA formation during IVT reaction.** Front. Mol. Biosci. 10, (2023).
8. Mu, X. & Hur, S. **Immunogenicity of *in vitro*-transcribed RNA.** Acc. Chem. Res. 54, 4012–4023 (2021).
9. Reshetnikov, V. et al. **Untranslated region sequences and the efficacy of mRNA vaccines against tuberculosis.** Int. J. Mol. Sci. 25, 888 (2024).
10. Beverly, M., Hagen, C. & Slack, O. **Poly A tail length analysis of *in vitro* transcribed mRNA by LC-MS.** Anal. Bioanal. Chem. 410, 1667–1677 (2018).
11. MEGAscript T7 Transcription Kit (Thermo). **https://www.trilinkbiotech.com/legal-notices.**
12. Zhang, J. et al. **Recent advances and innovations in the preparation and purification of *in vitro*-transcribed-mRNA-based molecules.** Pharmaceutics 15, 2182 (2023).
13. Camperi, J. et al. **Comprehensive impurity profiling of mRNA: Evaluating current technologies and advanced analytical techniques.** Anal. Chem. 96, 3886–3897 (2024).
14. Camperi, J. et al. **Exploring the Impact of *In Vitro*-Transcribed mRNA Impurities on Cellular Responses.** Anal. Chem. (2024).
15. Biziaev, N. et al. **The impact of mRNA poly(A) tail length on eukaryotic translation stages.** Nucleic Acids Res. 52, 7792–7808 (2024).
16. Arbuthnot, P., Ely, A. & Bloom, K. **A convenient method to generate and maintain poly(A)-encoding DNA sequences required for *in vitro* transcription of mRNA.** Biotechniques 66, 37–38 (2019).
17. Perenkov, A. D., Sergeeva, A. D., Vedunova, M. V. & Krysko, D. V. ***In vitro* transcribed RNA-based platform vaccines: Past, present, and future.** Vaccines (Basel) 11, 1600 (2023).
18. Molodtsov, V., Anikin, M. & McAllister, W. T. **The presence of an RNA: DNA hybrid that is prone to slippage promotes termination by T7 RNA polymerase.** J. Mol. Biol. 426, 3095–3107 (2014).
19. Tateishi-Karimata, H., Isono, N. & Sugimoto, N. **New insights into transcription fidelity: thermal stability of non-canonical structures in template DNA regulates transcriptional arrest, pause, and slippage.** PLoS One 9, e90580 (2014).
20. Polack, F. P. et al. **Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine.** N. Engl. J. Med. 383, 2603–2615 (2020).

ELEGEN | INNOVATION IS OUR **DNA**
3565 Haven Ave, Suite 3, Menlo Park, CA 94025