Cell-free, Linear IVT-ready DNA Accelerates mRNA Therapeutics Development

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Introduction

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 high-quality linear IVT-ready DNA templates with defined poly(A) lengths (70 - 130 bp).

Here we compare these linear templates to conventional plasmids in IVT reactions and evaluate the quality and functionality of the resulting mRNA.

Objectives

Compare the quality and functionality of cell-free, linear IVT-ready DNA templates with plasmid-derived templates and workflows:

- DNA templates QC specifications
- Performance in IVT reactions and characterization of the resulting mRNA
- *In vitro* functionality of the mRNA

Methods

Reporter Constructs and Transcription

mCherry and fLuc reporters were synthesized as linear DNA templates with encoded 90 or 130 bp poly(A) sequences. For this study we used T7 promoter and part of the 5′ UTR from *Homo sapiens* hemoglobin subunit alpha 1 as 5′ UTR. Equivalent plasmid-derived templates obtained from Supplier A were linearized prior to use. IVT reactions were performed using the MEGAscript™ T7 Kit (ThermoFisher).

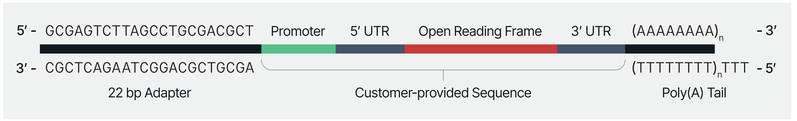
DNA templates and mRNA QC

Poly(A) tail length and distribution were assessed using PacBio sequencing. The yield, size, and integrity of mRNA products were evaluated by Nanodrop spectrophotometer, Agilent Bioanalyzer, and LC-MS analysis of T1 RNase digested poly(A) products.

Functional Assay

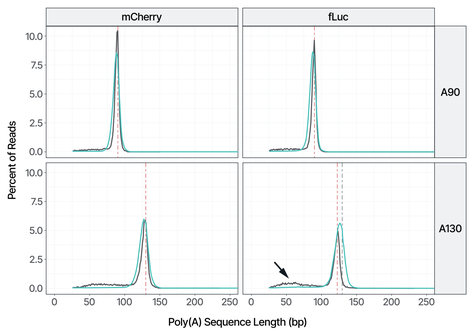
Capped fLuc mRNA (HiScribe CleanCap® AG, NEB) was translated *in vitro* using the 1-Step Human IVT Kit (Thermo). Luciferase activity was measured using the Pierce™ Glow Assay and VICTOR Nivo plate reader (PerkinElmer).

Linear IVT-ready template design



Results

Linear IVT-ready DNA templates demonstrate comparable to better poly(A) tail length and size distribution

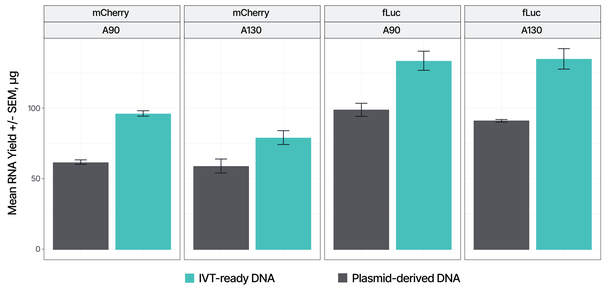


- PacBio sequencing demonstrates comparable poly(A) tail length and size distribution
- Higher frequency of molecules with shorter poly(A) tails was observed from linearized plasmids, particularly in the longer tailed templates
- fLuc plasmid from Supplier A carried a A123 poly(A) tail (longest available) compared with A130 for the linear IVT-ready template

Template Type:
IVT-ready DNA | Plasmid-derived DNA

Increased RNA output from linear IVT-ready templates vs. plasmids

IVT reactions using linear IVT-ready DNA produced an approximately 1.5-fold greater RNA yield when compared to an equivalent mass of linearized plasmid, across two different reporter genes each with two different poly(A) tail lengths.

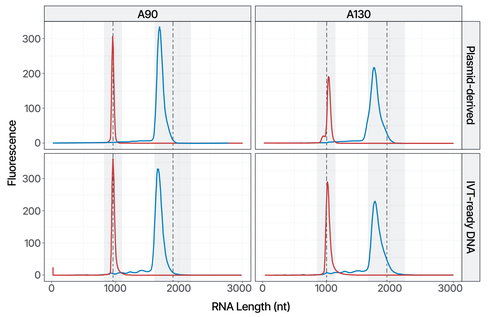


Bars represent mean ± SE, n = 3 - 6

500 ng DNA template, as measured by Qubit, were used in each reaction

RNA yield measured by Nanodrop spectrophotometer

Bioanalyzer traces demonstrate comparable size and integrity of RNA across IVT-ready and plasmid templates

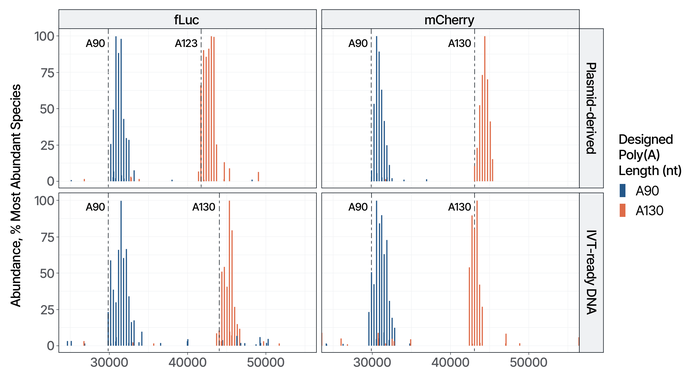


Traces demonstrate that RNA products show close agreement with the expected product size (dashed line, shaded box at +/- 15% expected size) and a single main peak.

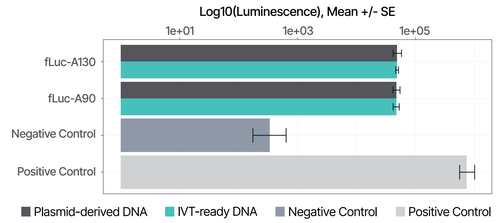
fLuc plasmid has an actual poly(A) tail of 123 nt, compared with 130 nt for IVT-ready template.

mCherry
fLuc

Comparable poly(A) tail lengths and polydispersity observed by LC-MS analysis of RNAse T1 digestion products



fLuc *in vitro* functionality test demonstrates comparable luminescence resulting from linear IVT-ready DNA & linearized plasmid templates



Bars represent mean ± SE, n = 3

CFPE reactions were initiated with 2.25 µg of RNA each and incubated at 30°C for 5 hours.

Positive control: 2.25 µg of CleanCap® Firefly Luciferase mRNA (TriLink Biotechnologies).

Negative control: no template

Summary

Linear IVT-ready DNA and plasmid-derived templates encoding fLuc and mCherry were tested in IVT reactions. Both linear templates harbored 90 and 130 bp poly(A) sequences, whereas only the mCherry plasmid carried a 130 bp sequence (fLuc plasmid: 123 bp), highlighting limitations in production of plasmids with long poly(A) sequences.

IVT-ready templates demonstrate comparable or superior IVT performance, with consistently higher or equivalent RNA yield per 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, and flexible alternative to plasmid-derived templates to accelerate mRNA therapeutic development.