Reducing double-stranded RNA formation in *in vitro* transcription using a novel RNA polymerase

A robust and versatile enzyme enabling seamless integration into RNA synthesis protocols

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Introduction

Double-stranded RNA (dsRNA) is a significant contaminant in mRNA. During *in vitro* transcription (IVT), the RNA polymerase transcribes template DNA to produce the desired RNA for downstream applications, such as a vaccine or other therapeutics. During IVT, RNA polymerases naturally generate dsRNA by products through a number of mechanisms including cryptic promoters or loopback RNAs. Once the RNAs are in cells or animals, these dsRNA contaminants can then trigger innate immune activation and diminish protein expression, thereby jeopardizing the safety, tolerability, and efficacy of the potential therapeutic product[1].

There are two primary strategies for mitigating dsRNA in mRNA products: removal after synthesis and prevention during synthesis (**Figure 1**). Post-synthesis removal of dsRNA impurities typically involves purification processes, most commonly ion-pair reverse-phase high-performance liquid chromatography (IP-RP-HPLC). Although HPLC effectively reduces dsRNA levels, this supplementary purification step can be time-consuming, result in yield losses of up to 50%, and impede scalability, making it challenging and expensive for large-scale mRNA production. Furthermore, purifying long sequences like self-amplifying RNAs (saRNAs) using HPLC can be more difficult without compromising their integrity.

Conversely, dsRNA byproducts can be minimized during IVT through optimization. For instance, design of experiments (DoE) can be employed to adjust IVT parameters such as buffer, pH, ions, nucleotides, enzyme concentration, and temperature — exemplified by TriLink's proprietary **CleanScript® method**[2]. However, these optimizations can require significant time and effort to achieve dsRNA reduction without negatively impacting yield or other critical quality attributes.

Recently, novel mutant enzymes have been developed to replace the commonly used wild-type T7 RNA polymerase to minimize dsRNA formation in IVT[3,4]. While theoretically simpler than the DoE approach, some enzymes may necessitate IVT condition optimization — especially when used in co-transcriptional capping — to avoid adverse effects on mRNA quality attributes such as yield, integrity, and capping efficiency. To address these challenges, TriLink BioTechnologies®, in collaboration with its sister company Alphazyme, has introduced a novel RNA polymerase — **CleanScribe™ RNA Polymerase** — that reduces dsRNA formation by up to 85% with simple integration into IVT for seamless mRNA synthesis.

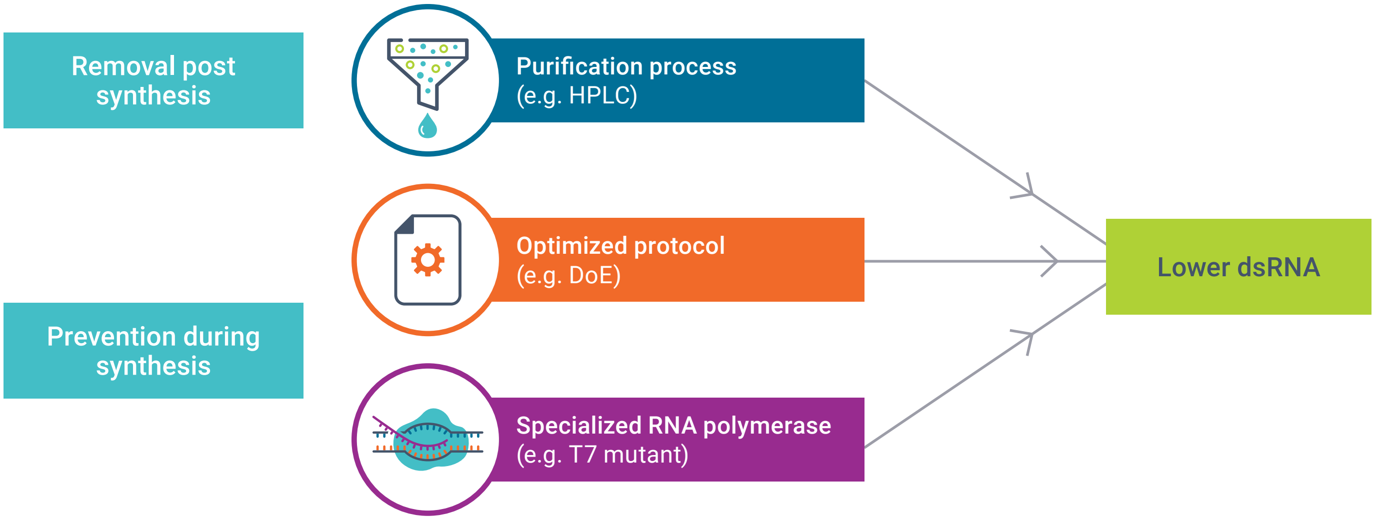

Figure 1. Common strategies to remove or reduce dsRNA from *in vitro*–transcribed mRNAs.

Significant reduction of dsRNA in co-transcriptionally capped mRNAs

To determine the level of dsRNA reduction, we compared CleanScribe RNA Polymerase and wild-type T7 RNA polymerase side-by-side in IVT with co-transcriptional capping. We tested them under the following conditions.

- **Detection of dsRNA by two methods:** J2 immunoblot and double-antibody M2/M5 sandwich ELISA (Vazyme kit)
- **Synthesis of three mRNA constructs of different sizes:** enhanced green fluorescent protein (EGFP, 1 kb), firefly luciferase (FLuc, 1.9 kb), and CRISPR-associated protein 9 (Cas9, 4.5 kb)
- **Co-transcriptional capping with two cap analogs: CleanCap® Reagent AG (3' OMe)** and **CleanCap® Reagent M6**

Under these conditions CleanScribe RNA polymerase reduces dsRNA formation by 50-80% relative to the wild-type T7 RNA polymerase (**Figure 2**).

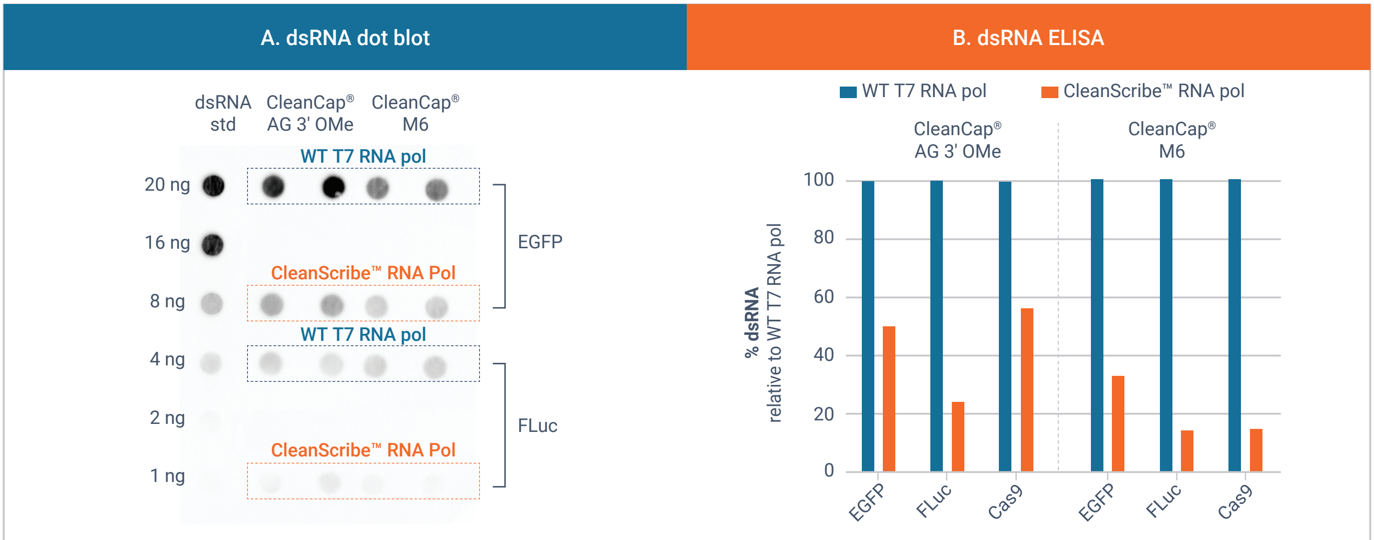

Figure 2. CleanScribe RNA Polymerase significantly reduces dsRNA formation during IVT. EGFP, FLuc, and Cas9 mRNAs were synthesized using either wild-type T7 (WT T7) or CleanScribe RNA polymerase with their respective described co-transcriptional cap analogs. Following LiCl precipitation purification, dsRNA levels were quantified by (A) J2 dot blot (with 2 µg of mRNA loaded each) and (B) dsRNA ELISA.

An optimal dsRNA reduction in IVT should not be detrimental to other desired quality attributes of mRNA such as yield, capping efficiency, and integrity. We assessed these critical quality attributes using CleanScribe™ RNA Polymerase and the wild-type T7 RNA Polymerase. Both show similar results, achieving 3-5 mg/mL of mRNA, >95% capping efficiency, and up to 80% integrity (**Figure 3**).

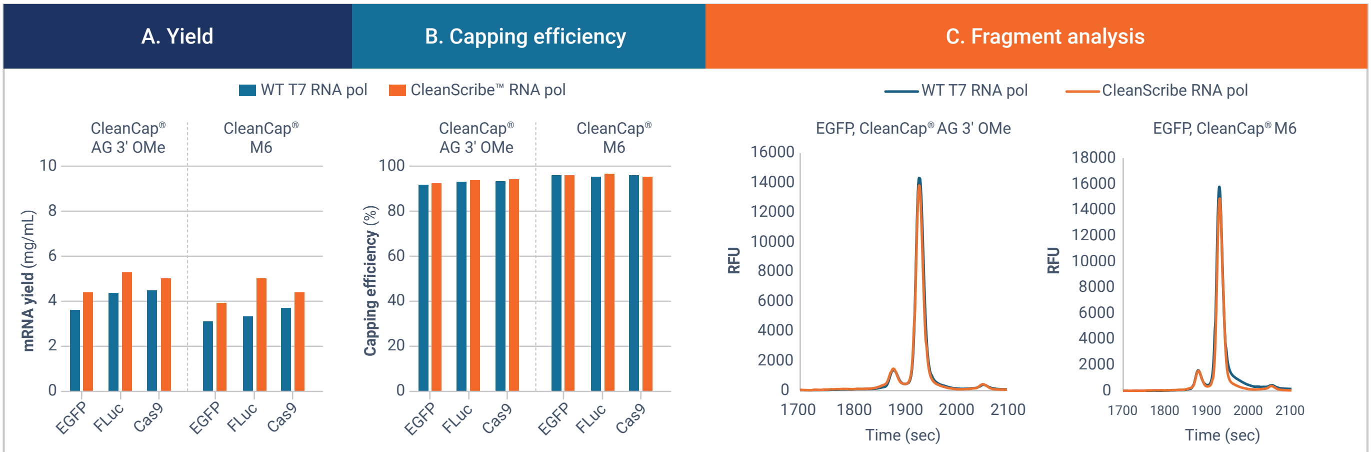

Figure 3. CleanScribe RNA Polymerase exhibits comparable mRNA yield, capping efficiency, and integrity to WT T7 RNA polymerase. Comparison of WT T7 and CleanScribe RNA polymerase for (A) mRNA yield measured by UV spectrometry, (B) capping efficiency determined by LC-MS, and (C) mRNA integrity assessed by fragment analysis after LiCl precipitation, with representative EGFP mRNA profiles shown.

Further reduction of dsRNA in IVT protocols established for low dsRNA

Next, we explored if CleanScribe RNA Polymerase could further reduce dsRNA in an IVT protocol already optimized for low dsRNA levels as well as increased yields. Substituting wild-type T7 RNA polymerase with CleanScribe RNA Polymerase in our proprietary CleanScript® protocol, which had been optimized with wild-type T7 RNA polymerase for dsRNA reduction, further reduced dsRNA by 70-80%, even to undetectable levels by immunoblot for FLuc in some cases. Importantly, using CleanScribe RNA Polymerase in the protocol maintained optimized yields of 8-10 mg/mL and did not negatively impact mRNA integrity or capping efficiency (**Figure 4**).

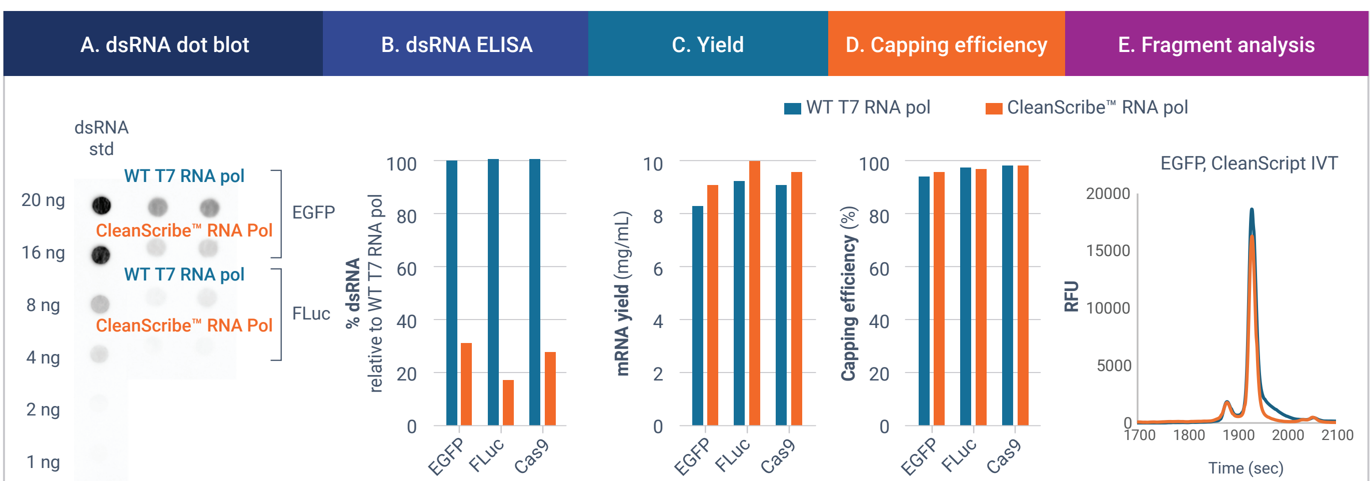

Figure 4. CleanScribe RNA Polymerase further minimizes dsRNA in IVT reactions optimized for low dsRNA and high yield. EGFP, FLuc, and Cas9 mRNAs incorporating CleanCap® AG 3'OMe were synthesized using WT T7 or CleanScribe RNA polymerase under TriLink's CleanScript IVT conditions. After LiCl precipitation, resulting mRNAs were analyzed for (A) dsRNA levels by J2 dot blot, (B) dsRNA levels by ELISA, (C) yield by UV spectrometry, (D) capping efficiency by LC-MS, and (E) integrity by fragment analysis with representative EGFP mRNAs shown.

Compatibility with using modified nucleotides in IVT

Modified nucleotides are increasingly used in the development of mRNA therapeutics to enhance the safety, efficacy, and stability of synthesized mRNAs. For example, the incorporation of chemically modified nucleotides, such as N1-methylpseudouridine for mRNA and 5-methylcytidine for saRNA, has been shown to decrease inflammatory responses, improve RNA stability, and enhance translational efficiency [5,6].

To ensure that CleanScribe™ RNA Polymerase could successfully incorporate modified nucleotides in RNA synthesis, we evaluated CleanCap® AG 3' OMe and CleanCap® M6 IVT reactions where uridines were substituted with N1-methylpseudouridines using the CleanScribe enzyme. As shown in **Figure 5**, dsRNA levels were reduced by up to 90% relative to the wild-type T7 RNA Polymerase across three mRNA constructs while maintaining mRNA yield and quality.

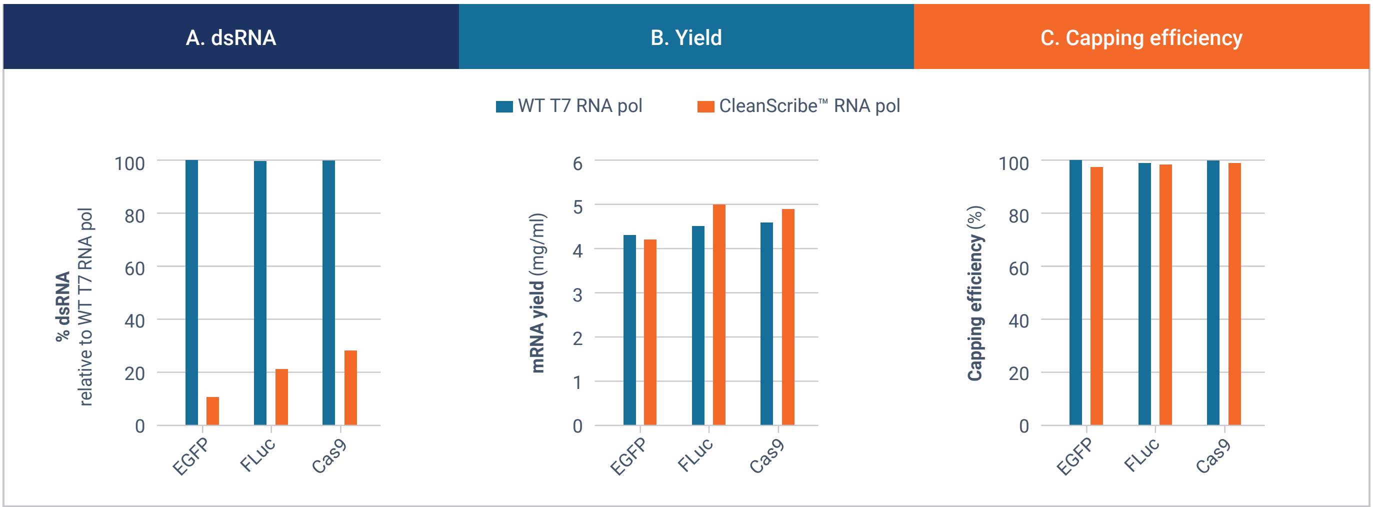

Figure 5. CleanScribe RNA Polymerase efficiently incorporates N1-methylpseudouridine. EGFP, FLuc, and Cas9 mRNAs containing CleanCap AG 3' OMe or CleanCap M6 and N1-methylpseudouridine (replacing uridine) were synthesized using WT T7 or CleanScribe RNA polymerase and purified by LiCl precipitation. The resulting mRNAs were analyzed for (A) dsRNA levels by ELISA, (B) yield by UV spectrometry, and (C) capping efficiency by LC-MS.

Integration of the enzyme into other existing IVT protocols

RNA polymerases that minimize dsRNA formation can have limitations, such as only functioning within specific IVT reaction conditions, necessitating optimization of existing protocols. To address this, we investigated the compatibility of CleanScribe RNA Polymerase as a direct substitute in various IVT protocols. Using five commercially available IVT kits with diverse properties, we replaced kit-provided RNA polymerases with CleanScribe RNA Polymerase and observed consistent dsRNA reduction with minimal impact on yields (**Figure 6**). Note that some protocols may include the enzyme mix that contains other enzymes like inorganic pyrophosphatase and RNase inhibitor. In that case, those enzymes may need to be supplemented to work properly when making the substitution.

A. Enzyme substitution

Provider	Co-transcriptional protocol with CleanCap analog?
Vendor A	Yes
Vendor B	Yes
Vendor C	No
Vendor D	No
Vendor E	No

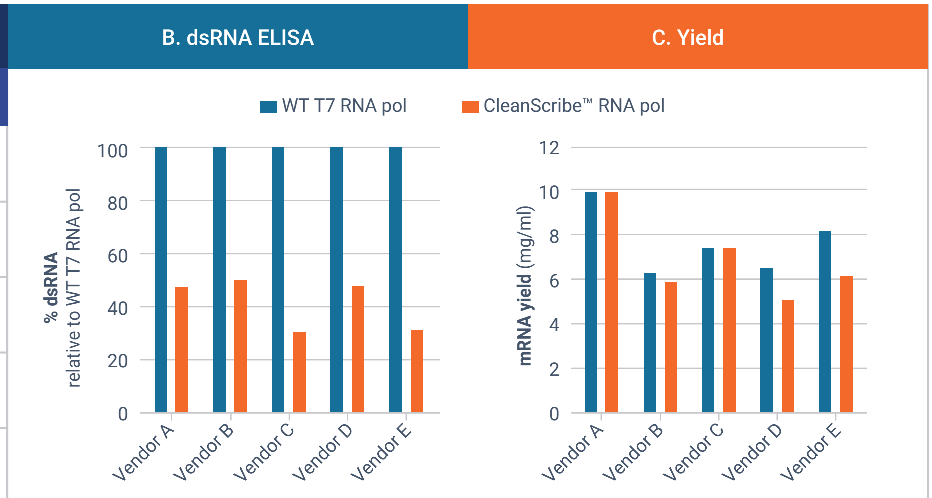

Figure 6. Substituting provided RNA polymerases with CleanScribe RNA Polymerase in existing IVT kits reduces dsRNA without compromising mRNA yield. (A) CleanScribe RNA Polymerase was directly substituted for RNA polymerases in five commercially available mRNA synthesis kits (with and without co-transcriptional capping). The resulting mRNAs were analyzed for (B) dsRNA and (C) mRNA yield.

Synthesis of long and complex templates (saRNAs) with modified nucleotides

saRNAs are becoming an attractive option for vaccine and therapeutic development due to their ability to replicate within cells, enabling higher protein expression at lower doses compared to mRNAs. However, synthesizing saRNAs by IVT poses challenges as their long and complex sequences can result in incomplete transcription, heterogenous capping, and high levels of dsRNA formation.

To investigate CleanScribe RNA Polymerase's capability to synthesize such long and complex templates, we examined FLuc and mCherry saRNA templates that are up to 10 kb long. We used CleanCap® AU cap analog which carries the authentic 5' cap of alphavirus backbone of saRNAs[7]. We also tested its ability to incorporate 5-methylcytidine (5mC), a popular modified nucleotide used in saRNA. We demonstrated that CleanScribe RNA Polymerase successfully synthesizes saRNAs with up to 88% integrity as the fragment analysis data showed while reducing dsRNA formation and maintaining overall yield (**Figure 7**).

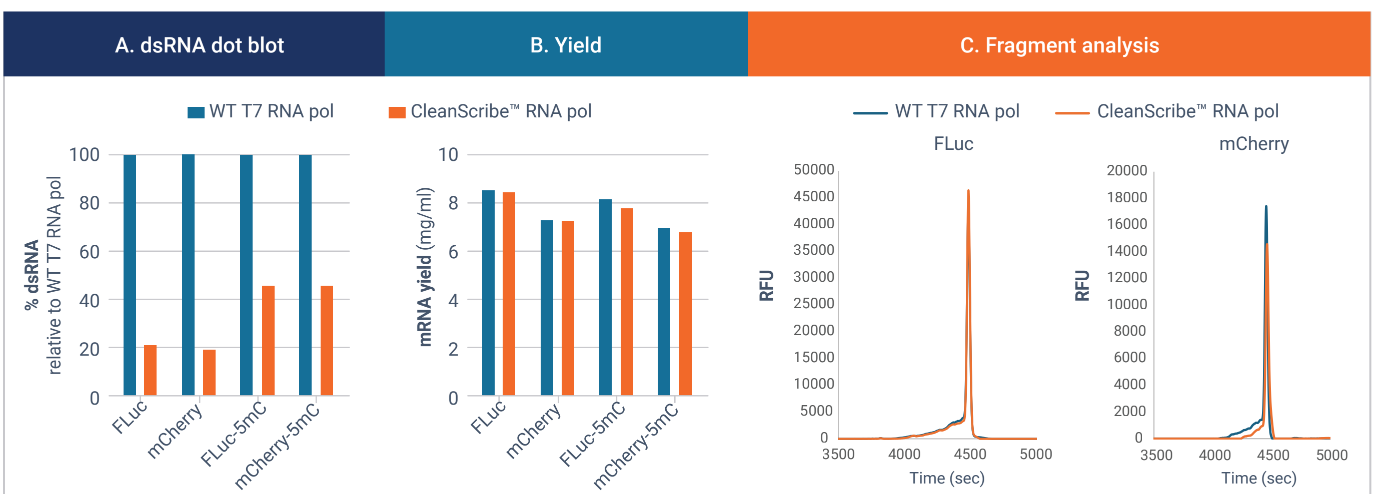

Figure 7. CleanScribe RNA Polymerase reduces dsRNA formation during saRNA synthesis. FLuc and mCherry saRNAs (either unmodified or with 5mC replacing C) with CleanCap AU were synthesized using WT T7 or CleanScribe RNA polymerase. The saRNAs were analyzed for (A) dsRNA levels by dot blot, (B) yield by UV spectrometry, and (C) integrity by fragment analysis after silica-membrane purification with representative unmodified saRNAs shown.

dsRNA reduction leads to an increase in mRNA potency

dsRNA impurities can trigger adverse inflammatory responses and compromise mRNA translation into protein, reducing therapeutic tolerability and efficacy. To assess the effect of dsRNA reduction, we measured innate immune activation and protein expression in a reporter cell line using mRNA synthesized with either CleanScribe RNA Polymerase or wild-type T7 RNA polymerase.

We transfected the synthesized CleanCap M6 EGFP mRNAs into A549-Dual® cells and measured Lucia® luciferase expression, which is under the control of interferon-stimulated gene 54 (ISG54) promoter, as a readout for innate interferon responses. We used two positive controls to trigger immune responses: (1) 3p-hpRNA, an 89-mer synthetic RNA oligo of an influenza A virus with a hairpin structure containing an uncapped 5' triphosphate and (2) Poly(I:C), a mismatched synthetic dsRNA polymer mimicking viral dsRNA. We also examined EGFP as a readout for protein expression.

mRNA synthesized using CleanScribe RNA Polymerase resulted in lower innate inflammatory response and higher protein expression than wild-type T7 enzyme–synthesized mRNA (**Figure 8**). Together, these findings demonstrate that implementation of CleanScribe RNA Polymerase for dsRNA reduction in IVT can increase mRNA potency through reduced immunogenicity and increased protein expression.

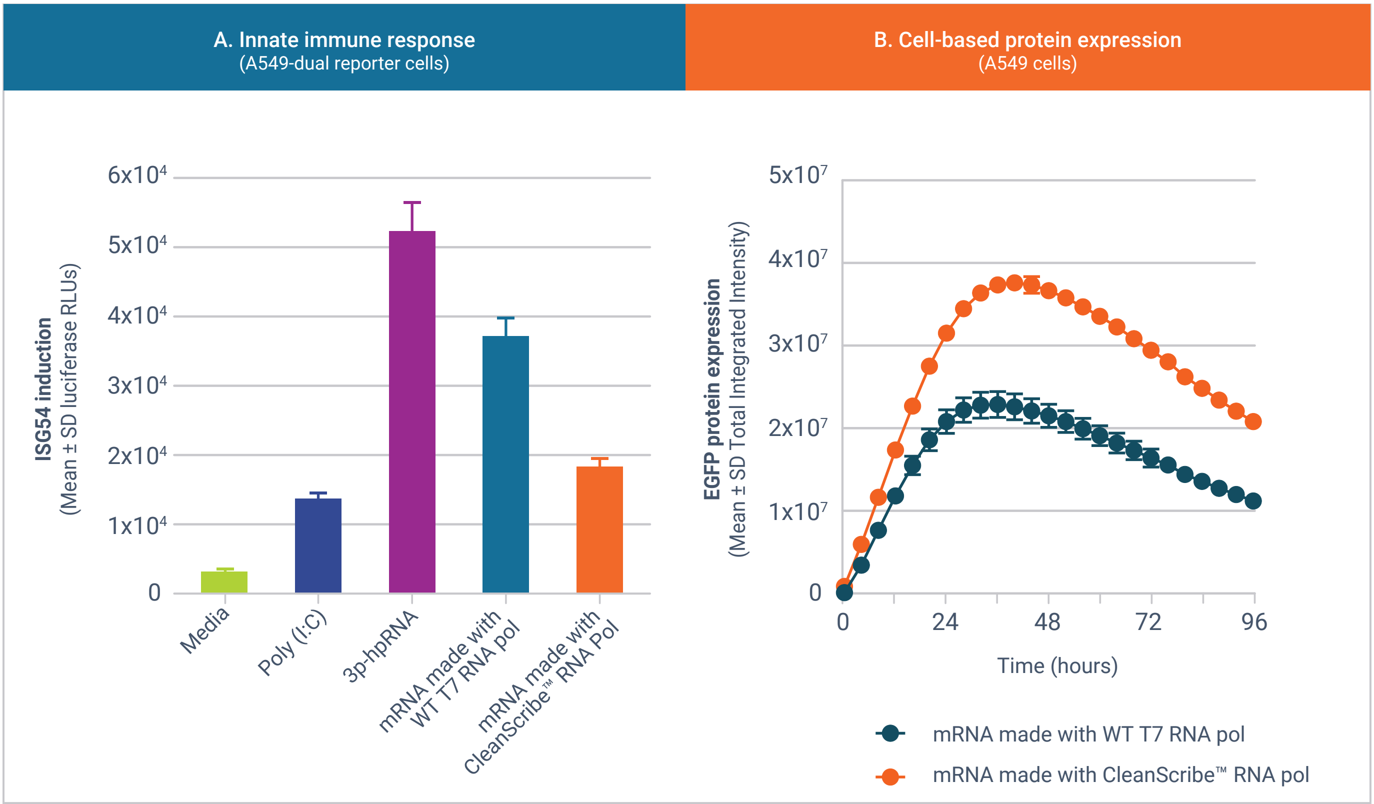

Figure 8. Reduced dsRNA levels result in increased mRNA potency *in vitro*. EGFP mRNAs with CleanCap M6 synthesized using WT T7 or CleanScribe RNA Polymerase were transfected into A549-Dual cells. (A) Innate interferon response was assessed by measuring ISG54-driven Lucia luciferase expression in cell supernatants 24 hrs post-transfection. (B) mRNA-encoded GFP protein expression in A549 cells was monitored over time by fluorescence. All conditions were performed in triplicate.

Conclusion

Minimizing dsRNA byproducts in mRNA therapeutics is crucial for their safety and efficacy, as dsRNA contamination can trigger unwanted inflammatory responses, reduce protein expression, and compromise therapeutic potency. While purification can remove dsRNA, this process is time-consuming and can negatively impact mRNA yield.

Our CleanScribe RNA Polymerase offers a simple yet effective solution for dsRNA reduction during IVT. Characterization studies outlined in this tech note demonstrate its ability to:

- Reduce dsRNA formation across various constructs, CleanCap cap analogs, and modified nucleotides
- Directly replace wild-type T7 RNA polymerase in diverse IVT protocols without compromising other critical quality attributes
- Efficiently synthesize long and complex templates like saRNAs while minimizing dsRNA formation
- Improve mRNA performance by lowering innate inflammatory responses and increasing protein expression

These benefits translate to not only increased therapeutic mRNA potency but also reduced manufacturing costs for drug developers by streamlining the process and eliminating additional dsRNA reduction steps. As the mRNA field advances, innovations like CleanScribe RNA Polymerase that address dsRNA contamination will be essential for expanding the full potential of mRNA therapeutics.

References

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