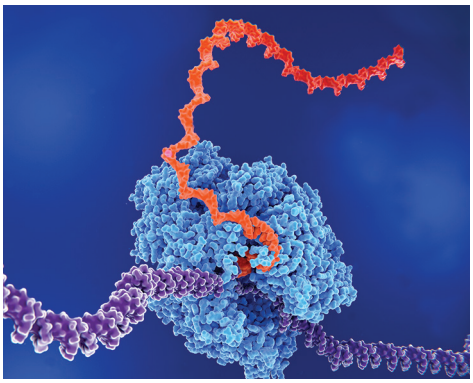

High efficiency mRNA synthesis and ultra-low immunogenicity achieved with the T7 mScript™ Complete mRNA Production Systems

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Introduction

Messenger RNA (mRNA)-based therapeutics and vaccines have emerged as a transformative platform in molecular medicine, offering rapid, scalable, and cell-free production of biologically active proteins.[1] The success of mRNA vaccines during the COVID-19 pandemic has accelerated interest in optimizing mRNA synthesis, modification, and purification to enhance translational efficiency and minimize innate immune activation. *In vitro* transcription (IVT) using bacteriophage T7 RNA polymerase is a widely adopted method for producing synthetic mRNA. However, IVT reactions often generate double-stranded RNA (dsRNA) byproducts, which can trigger potent antiviral responses through pattern recognition receptors such as RIG-I and MDA5.[2-4] These immune responses can reduce protein expression and compromise the safety and efficacy of mRNA-based therapeutics. To address this, chemical modifications such as N1-methyl-pseudouridine (N1meΨ) substitution for uridine, and post-transcriptional purification strategies to remove dsRNA, have been employed to reduce immunogenicity and improve mRNA stability and translation.[5-9]

In this study, we synthesized firefly luciferase mRNA using the T7 mScript™ Complete mRNA Production Systems, incorporating either canonical UTP or modified N1meΨTP. We evaluated the impact of dsRNA removal using the included Min-Immune™ Gold dsRNA Removal Kit module on mRNA quality and expression potential. Quality control assays were performed to assess capping efficiency, poly(A) tail length, and overall mRNA integrity. This work contributes to the growing body of knowledge aimed at refining mRNA production workflows for research and therapeutic applications and positions the T7 mScript™ Complete mRNA Production Systems as highly suitable for synthesis of high-quality mRNA devoid of dsRNA and amenable for use in downstream applications such as mRNA vaccine development and cell and gene therapy research.

The Challenge

Unmodified mRNA can result in mRNA that has lower stability, activates the immune system, and reduced translational efficiency. Additionally, immune responses caused by dsRNA byproducts generated during *in vitro* transcription can compromise the safety and efficacy of mRNA-based therapeutics which is a major challenge for downstream mRNA therapeutic applications.

The Solution

We present an optimized dual strategy to create high-yield single-stranded mRNA using mRNA modified with N1-methyl-pseudouridine (N1meΨ) that produces markedly higher protein expression using the INCOGNITO™ T7 mScript™ Complete N1meΨ-mRNA Production System compared to unmodified transcripts. The removal of double-stranded RNA contaminants using the Min-Immune™ Gold dsRNA Removal Kit procedure further improves protein expression, and significantly reduces immunogenicity. This integrated dual workflow seamlessly combines high-efficiency mRNA transcription with N1meΨ modification and dsRNA removal, providing an optimal solution for generating ultra-low immunogenicity mRNA for downstream applications.

Materials and Methods

In vitro Transcription of Firefly Luciferase mRNA

Firefly luciferase mRNA was synthesized using the T7 mScript™ Complete Standard mRNA Production System using canonical nucleotides (Cat. No. MSCC250225) and the INCOGNITO™ T7 mScript™ Complete N1meΨ-mRNA Production System using N1-methyl-pseudouridine-5′-triphosphate [N1meΨTP] (Cat. No. IMCMY250225) following the manufacturer's protocol. Each reaction was carried out at 37°C for 30 minutes using a linearized DNA template encoding the firefly luciferase gene.

Following transcription, the IVT RNA was purified by ammonium acetate (NH_4OAc) precipitation, washed with 70% ethanol, resuspended in RNase-free water, and quantified by spectrophotometry to confirm yield and purity. Post-transcriptional modifications were performed sequentially according to the kit instructions. 5′-end capping reactions were performed to generate a Cap 1 structure, followed by 3′-end polyadenylation. The final mRNA product, approximately 1.8 kilobases in length, was purified again and quantified by spectrophotometry to confirm yield and purity.

Double-Stranded RNA (dsRNA) Removal

To assess the impact of dsRNA on mRNA expression, an aliquot of untreated mRNA was reserved as a control for presence and quantity of contaminating dsRNA which is produced during the transcription reaction. Double-stranded RNA is a molecule composed of two complementary strands of RNA, held together by hydrogen bonds. It is a common contaminant produced during IVT. This dsRNA is unwanted because it triggers innate immune responses and reduces the efficiency of protein translation. The remaining mRNA was subjected to dsRNA removal using the Min-Immune™ Gold dsRNA Removal module included in the kits according to the kit instructions. Briefly, RNA was incubated with the kit reagents at 37°C for 1 hour. Following treatment, RNA was purified by NH_4OAc precipitation, washed with 70% ethanol, resuspended in RNase-free water, and quantified by spectrophotometry.

mRNA Quality Assessment

The integrity and quality of the synthesized mRNA were evaluated using CELLSCRIPT™'s EZ-QC™ mRNA quality control assay kits. These included the EZ-QC™ mRNA Cap 1 Efficiency Assay (Cat. No. ONE240910), the EZ-QC™ mRNA Capping Efficiency Assay Kit (Cat. No. ACE240910), and the EZ-QC™ mRNA Poly(A) Tail Length Assay Kit (Cat. No. PAT240910). All assays were performed in accordance with the manufacturer's protocols.

Table 1. mRNA Quality Assessment with EZ-QC™ Kits.

		Assay Results	
mRNA Quality Assay	**Catalog #**	**UTP**	**N1meΨTP**
EZ-QC™ mRNA Capping Efficiency Assay	ACE240910	98% Capped	95% Capped
EZ-QC™ mRNA Cap 1 Efficiency Assay	ONE240910	94% Cap 1	93% Cap 1
EZ-QC™ mRNA Poly(A) Tail Length Assay	PAT240910	148-A's	166-A's

Luciferase Transfection and Assay Methods

The firefly luciferase mRNAs were transfected in duplicate into human THP-1 cells (ATCC), a monocytic cell line. The cells were expanded in serum-containing media and plated 24 hours prior to transfection. $2x10^5$ cells were plated per well and transfected in duplicate with 1.5 µg of each mRNA and 2.5 µl Lipofectamine™ MessengerMax™ Transfection Reagent (Thermo Fisher Scientific) in 100 µl of Opti-MEM™ medium (Thermo Fisher Scientific). After a 24-hour incubation at 37°C with 5% CO_2, the cells were collected, washed, and lysed with Reporter Lysis Buffer (Promega). The lysates were assayed with an equal volume of ONE-Glo™ EX Luciferase Assay Substrate (Promega). Average relative light units (RLUs) from duplicate assays on duplicate samples were plotted.

RT-qPCR to detect Innate Immune Response Methods

Human THP-1 cells were transfected in duplicate as described. After a 24-hour incubation, the cells were collected, washed and total cellular RNA was isolated from each well using the MasterPure™ Complete DNA and RNA Purification Kit (Biosearch Technologies). Equivalent amounts of RNA were amplified by RT-qPCR to detect differences in the cellular response to mRNA transfection. The purified cellular RNA was reverse transcribed and amplified using the iTaq Universal SYBR® Green One-Step Kit (Bio-Rad) with 9 primer sets using the Bio-Rad CFX 96 real-time cycler. The results from the test samples were averaged, normalized to GAPDH expression, and presented relative to the results from control cells transfected without any mRNA. The relative normalized expression or $\Delta\Delta C_q$ was plotted for each primer set with the SEM.

Results

Luciferase Assay to Determine Protein Expression with N1meΨ-Modified mRNA

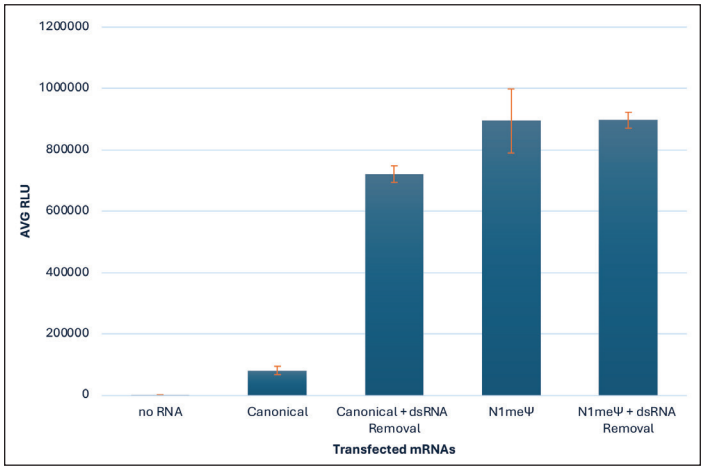

Figure 1. Measurement of luciferase activity in THP-1 cells 26 hours post-transfection. The combination of N1meΨ modification and dsRNA removal resulted in the highest luciferase expression.

N1meΨ-modified mRNA yielded the highest luciferase expression, with an average of ~895,000 RLU (Figure 1). Notably, dsRNA removal using the Min-Immune™ Gold module significantly enhanced expression of canonical unmodified mRNA (~721,000 RLU), approaching levels observed with N1meΨ-modified canonical mRNA to canonical mRNA +dsRNA removal, there was a 8.87 fold increase or a 887% increase in luciferase expression. The combination of N1meΨ modification and dsRNA removal resulted in the highest expression (~898,000 RLU), suggesting additive benefits of chemical modification and purification. Min-Immune™ Gold purified luciferase mRNA samples demonstrated significantly reduced amounts of dsRNA vs controls, with implications for highly improved translation of mRNA produced with canonical nucleotides.

RT-qPCR to Detect Innate Immune Response

Primer pairs were designed to RNA-responsive immune sensor genes including toll like receptors (TLR3, TLR7), dsRNA receptors (RIG-I, MDA5), interferon beta (IFNB1), nucleic acid receptors stimulated by the interferon response (OAS1, PKR) and a proinflammatory cytokine (TNF). These and other genes, when elevated, indicate transfected RNA has been detected by the cell. The stronger the response or expression of these genes, the larger the cascades of type one interferons, protein translation inhibition, RNA degradation and cell death.

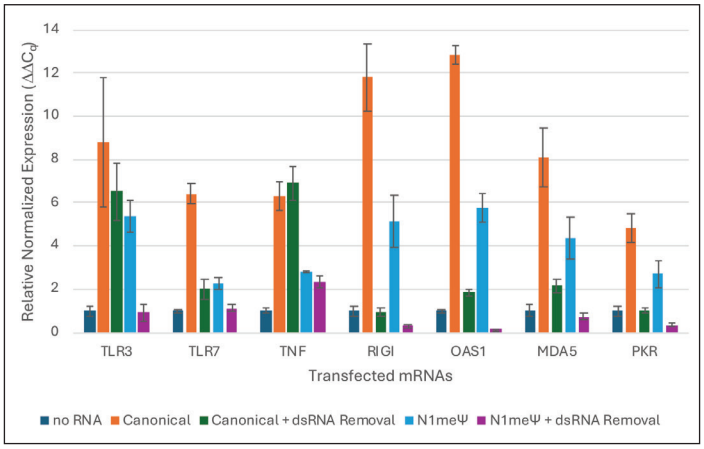

Figure 2. RT-qPCR measurement of relative normalized expression of RNA-responsive immune sensor genes. Canonical unmodified mRNA had the highest expression of immune sensors, while N1meΨ modified mRNA and dsRNA mRNA had the lowest.

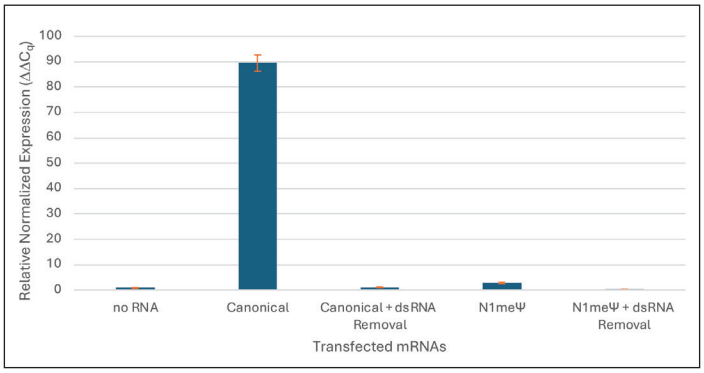

Figure 3. RT-qPCR relative normalized expression of innate immune response genes. Canonical mRNA had the highest immune response while N1meΨ mRNA in combination with dsRNA removal had the lowest innate immune response.

N1meΨ mRNA that has been purified with the Min-Immune™ Gold module of the kit results in almost no detectable innate immune response.

Conclusion

The results of this study demonstrate that both uridine analogs and post-transcriptional purification significantly influence the translational efficiency and immunogenicity of synthetic mRNA in human immune cells. Using firefly luciferase as a reporter, and the INCOGNITO™ T7 mScript™ Complete N1meΨ-mRNA Production System, we observed that mRNA modified with N1meΨ produced markedly higher protein expression compared to unmodified transcripts. This enhancement is consistent with previous reports showing that N1meΨ incorporation reduces innate immune recognition and improves ribosomal engagement, thereby increasing translation efficiency.

Importantly, the removal of dsRNA contaminants using the Min-Immune™ Gold purification procedure significantly improved luciferase expression by 887%, particularly for unmodified mRNA. This suggests that dsRNA byproducts generated during *in vitro* transcription are a major source of translational inhibition, likely through activation of innate immune sensors such as RIG-I and MDA5. The additive effect observed when combining N1meΨ modification with dsRNA removal underscores the value of a dual strategy to optimize mRNA performance.

RT-qPCR analysis revealed that unmodified mRNA strongly activated innate immune genes, including TLR3, RIG-I, OAS1 and IFNB1 indicating a robust immune response. In contrast, mRNA modified with N1meΨ triggered a much weaker immune response. When N1meΨ-modified mRNA was also purified to remove dsRNA, immune activation was nearly eliminated. These results show that unmodified or impure mRNA can stimulate immune pathways, but chemical modification and purification are effective strategies to reduce this response.

Together, these results support the use of the INCOGNITO™ T7 mScript™ Complete N1meΨ-mRNA Production System that incorporates N1meΨ and rigorous purification protocols free of dsRNA contamination to enhance the safety and efficacy of synthetic mRNA. This has important implications for the development of mRNA-based therapeutics and vaccines, particularly in contexts where immune activation must be minimized, such as protein replacement therapies or gene editing applications.

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