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RNA for Translation in Cells

Dual Optimization of Synthetic mRNA: Enhancing Translation and Minimizing Immunogenicity

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Abstract & Introduction

Messenger RNA (mRNA)-based therapeutics offer a versatile platform for rapid, scalable, and cell-free protein production. However, their clinical potential is often limited by innate immune activation and suboptimal translational efficiency. These issues largely stem from double-stranded RNA (dsRNA) byproducts generated during *in vitro* transcription (IVT) and the recognition of exogenous RNA by Toll-like receptors. To overcome these challenges, we implemented a dual-optimization strategy to produce ultra-low immunogenicity mRNA. This approach combines transcription using modified uridine analogs with downstream dsRNA removal via our proprietary enzymatic solution. To evaluate the effectiveness of this strategy, Firefly luciferase mRNA was synthesized using either canonical UTP or modified uridine analog triphosphates, with or without dsRNA purification. Quality control confirmed high capping efficiency, appropriate poly(A) tail length, and intact mRNA.

When transfected into human cells, mRNA containing modified uridine analogs significantly enhanced protein expression compared to unmodified transcripts. Furthermore, dsRNA removal using our proprietary enzymatic solution led to markedly amplified luciferase expression, underscoring the translational inhibition caused by dsRNA contaminants. RT-qPCR analysis revealed robust activation of innate immune genes (TLR3, RIG-I, OAS1, IFNB1) by unmodified mRNA. In contrast, mRNA containing modified uridine analogs elicited a substantially attenuated immune response. When combined with enzymatic dsRNA removal, immune activation was nearly abolished. These findings demonstrate that pairing chemical uridine modification with enzymatic dsRNA removal yields high-quality, immunogenicity-free mRNA, enabling robust mRNA-based applications where immune evasion is critical.

Materials and Methods

In vitro Transcription of Firefly Luciferase mRNA

Firefly luciferase mRNA was synthesized using the T7 mScript™ Complete Standard mRNA Production System (Cat. No. MSCC250225; uses canonical nucleotides), INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System (Cat. No. IMCY250125; uses pseudouridine-5′-triphosphate [ΨTP]), or INCOGNITO™ T7 mScript™ Complete N1meΨ-mRNA Production System (Cat.No. IMCMY250225; uses N1-methyl-pseudouridine-5′-triphosphate [N1meΨTP]) following the manufacturer's protocols. Each transcription 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

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. 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. 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, mRNA was incubated with the kit reagents at 37°C for 1 hour. Following treatment, mRNA 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 Capping Efficiency Assay Kit (Cat. No. ACE240910), the EZ-QC™ mRNA Cap 1 Efficiency Assay Kit (Cat. No. ONE240910), 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.

Luciferase Transfection and Assay Methods

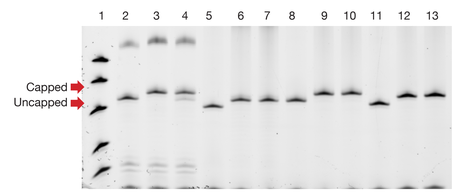
The firefly luciferase mRNAs were transfected in duplicate into JAWSII cells (ATCC), a mouse immature dendritic cell line. The cells were expanded in serum-containing media and plated 24 hours prior to transfection. $2x10^5$ cells were plated per well and transfected in duplicate with 1.5 µg of each mRNA and 2.5 µl Lipofectamine™ MessengerMax™ Transfection Reagent (Thermo Fisher Scientific) in 100 µl of Opti-MEM™ medium (Thermo Fisher Scientific). After a 26-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 (ATCC, monocytes) were transfected in duplicate as described. After a 26-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 ΔΔCq was plotted for each primer set with the SEM.

mRNA Quality Testing

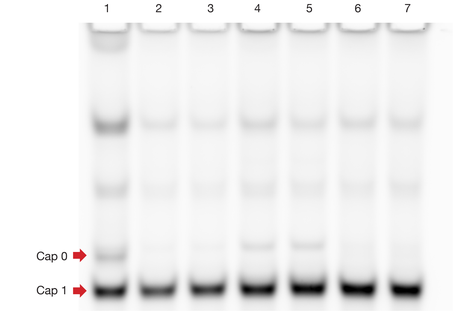
EZ-QC™ mRNA Capping Efficiency Assay Results (ACE240910)



Gel Image A: Results from EZ-QC™ mRNA Capping Efficiency Assay Kit Testing

Lane	Sample		% Capped
1	Oligo 10/60 Ladder		---
2	Un-capped Control		---
3	Capped (Cap - 0) Control		---
4	80/20 Control		80%
5	Canonical	IVT (un-capped)	0%
6		Capped mRNA	100%
7		Capped/Min-Immune™ Gold Treated mRNA	100%
8	Ψ	IVT (un-capped)	0%
9		Capped mRNA	99%
10		Capped/Min-Immune™ Gold Treated mRNA	99%
11	N1meΨ	IVT (un-capped)	0%
12		Capped mRNA	100%
13		Capped/Min-Immune™ Gold Treated mRNA	100%

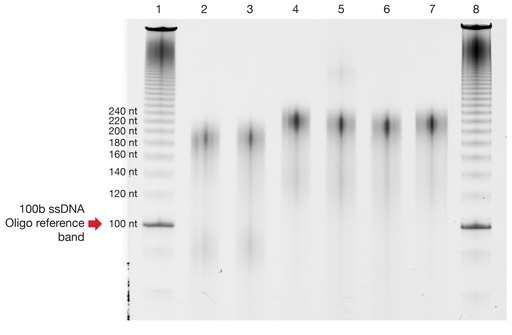
EZ-QC™ mRNA Cap 1 Efficiency Assay Results (ONE240910)



Gel Image B: Results from EZ-QC™ mRNA Cap1 Efficiency Assay Kit Testing

Lane	Sample		% Cap 1
1	80/20 Control		79.9 %
2	Canonical	Capped/Tailed mRNA	97.8 %
3		Capped/Min-Immune™ Gold Treated mRNA	97.9 %
4	Ψ	Capped/Tailed mRNA	94.5 %
5		Capped/Min-Immune™ Gold Treated mRNA	94.5 %
6	N1meΨ	Capped/Tailed mRNA	97.9 %
7		Capped/Min-Immune™ Gold Treated mRNA	97.3 %

EZ-QC™ mRNA Poly(A) Tail Length Assay Results (PAT240910)



Gel Image C: Results from EZ-QC™ mRNA Poly(A) Tail Length Assay Kit Testing

Lane	Sample		Tail Length
1	Poly(A) 20-mer Ladder		---
2	Canonical	Untreated mRNA	186-A's
3		Min-Immune™ Gold Treated mRNA	184-A's
4	Ψ	Untreated mRNA	213-A's
5		Min-Immune™ Gold Treated mRNA	201-A's
6	N1meΨ	Untreated mRNA	191-A's
7		Min-Immune™ Gold Treated mRNA	195-A's
8	Poly(A) 20-mer Ladder		---

Luciferase Assay to Determine Protein Expression with N1meΨ-Modified and Ψ-Modified mRNA

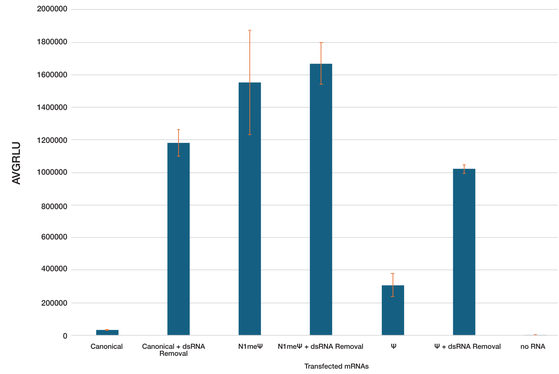


Figure 1. Measurement of luciferase activity in JAWSII cells 26 hours post-transfection. The combination of N1meΨ modification and dsRNA removal resulted in the highest luciferase expression.

N1meΨ-modified mRNA showed the highest luciferase expression pre-removal of dsRNA, averaging ~1,553,000 RLU. Notably, dsRNA removal using the Min-Immune™ Gold module significantly enhanced expression of canonical unmodified mRNA (~1,181,000 RLU), and Ψ-modified mRNA (1,021,000), each approaching levels observed with N1meΨ-modified mRNA. The combination of N1meΨ modification and dsRNA removal resulted in the highest expression (~1,668,000 RLU), demonstrating the additive benefits of both chemical modification and purification. Min-Immune™ Gold purified luciferase mRNA samples with significantly reduced amounts of dsRNA vs controls demonstrates highly improved translation of mRNA produced with and without uridine modification.

RT-qPCR to Detect Innate Immune Response

Primer pairs were designed to RNA-responsive immune sensor messages 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.

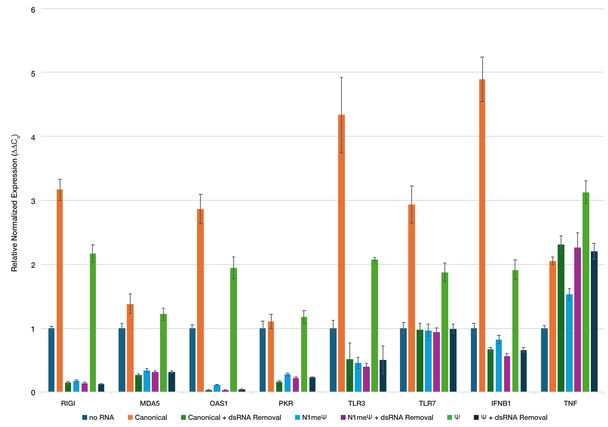


Figure 2. RT-qPCR measurement of relative normalized expression of RNA-responsive immune sensor genes. Canonical unmodified mRNA had the highest expression of immune sensors, while Ψ-modified mRNA also had a detectable response and N1meΨ-modified mRNA had the lowest response before dsRNA removal.

Using the Min-Immune™ Gold module for removal of dsRNA yields mRNA with minimal detectable activation of the innate immune system.

Conclusion

This study demonstrates that both uridine analog incorporation and dsRNA removal significantly enhance the translational efficiency and reduce the immunogenicity of synthetic mRNA in human immune cells. Using firefly luciferase as a reporter, mRNA synthesized with the INCOGNITO™ T7 mScript™ Complete N1meΨ-mRNA Production System showed markedly higher protein expression than unmodified transcripts. This aligns with prior findings that N1-methyl-pseudouridine (N1meΨ) reduces innate immune recognition and improves ribosomal engagement. Pseudouridine (Ψ) has a similar impact, enhancing translational efficiency and reducing immunogenicity, though to a lesser degree than N1meΨ, but still significantly better than canonical uridine-containing transcripts.

Critically, dsRNA removal using the Min-Immune™ Gold purification module enhanced luciferase expression 33.7-fold in unmodified mRNA, indicating that dsRNA byproducts are a major source of translational inhibition, likely via activation of innate immune sensors such as RIG-I and MDA5. The combination of N1meΨ modification and dsRNA removal yielded the highest expression levels, highlighting the additive benefits of this dual strategy.

RT-qPCR analysis revealed that unmodified mRNA strongly induced innate immune genes (TLR3, RIG-I, OAS1, IFNB1), while modified mRNA triggered a significantly weaker response. When combined with dsRNA removal, immune activation was nearly abolished.

These findings support the use of N1meΨ-modified, dsRNA-free mRNA to improve the safety and efficacy of mRNA therapeutics, particularly in applications where immune activation must be minimized, such as vaccines, protein replacement therapies, and gene editing.