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RNA for Translation in Cells

Enzymatic Elimination of dsRNA Byproducts Enhances Translation and Reduces Innate Immune Activation of mRNA

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Introduction

Messenger RNA (mRNA) technologies continue to show transformative potential across therapeutic fields, including engineered cell therapies and cell reprogramming, where precise control over immunogenicity is critical for success. Double-stranded RNA (dsRNA) species generated as unintended byproducts of *in vitro* transcription (IVT) represents a major focus of IVT derived RNA therapeutics. These dsRNA contaminants are potent activators of innate immune pathways, triggering pattern recognition receptors that suppress mRNA translation and compromise cellular viability.

Effects of dsRNA and Importance of dsRNARemoval

Double-stranded RNA (dsRNA) contaminants from *in vitro* transcription trigger innate immune responses and impair translation, making their removal critical for therapeutic RNA applications. Well-characterized sensors such as RIG-I, MDA5, TLR3, PKR and OAS interact with dsRNA and can be broadly grouped into inflammatory signaling and translation suppression pathways.

Acceptable thresholds for dsRNA removal vary by application, but dsRNA is an extremely potent immune activator even at very low levels. Internal data indicates that RIG-I can still be activated at dsRNA levels on the order of ~0.05% (%dsRNA of total mRNA in sample) in a single dose of N1meΨ-containing IVT mRNA, underscoring how little dsRNA is needed to engage antiviral pathways despite having immunomodulating nucleotide modifications.

Because interferon induction and downstream signaling place cells into an antiviral state, repeated dosing of mRNA that contains dsRNA contaminants is expected to be progressively less tolerated. This compounds the risk of translational shutoff and cell death in applications requiring multiple mRNA doses.

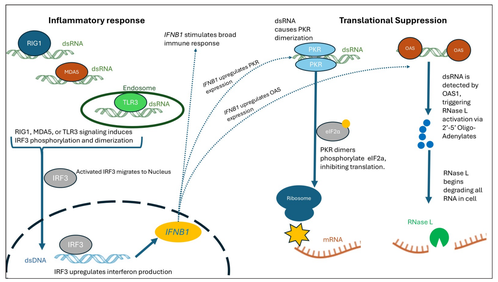

Figure 1. Inflammatory response and translation suppression mechanisms activated by dsRNA.

Purification Methods for dsRNA Removal

Generated double-stranded RNA (dsRNA) byproducts are similar in composition to desired RNA, necessitating specialized purification steps to selectively remove these immunogenic species. Multiple orthogonal approaches are now used to reduce dsRNA to levels compatible with therapeutic applications, particularly for systemic and repeated dose mRNA delivery.

Processes upstream of purification can strongly influence dsRNA formation. Strategies such as optimizing IVT conditions, refining template design and selecting alternative polymerases can lower dsRNA generation before purification. These measures, together with nucleoside modification (for example, N1-methyl-pseudouridine), are now standard practice, but they generally do not eliminate dsRNA on their own, especially for *in vivo* therapeutic or vaccine applications where innate immune activation must be tightly controlled. For many therapeutic use cases, dedicated dsRNA removal steps beyond uridine substitution and workflow optimization are therefore required to achieve the desired safety and expression profiles.

Chromatographic purification remains the most common tool to facilitate dsRNA removal. Cellulose-based chromatography exploits the preferential binding of dsRNA to cellulose in the presence of ethanol, allowing single-stranded mRNA to remain in the flow through and efficiently removing dsRNA species longer than approximately 30–80 bp. Reverse-phase HPLC separates RNA species based on hydrophobicity and conformation, enriching for full-length, properly-folded mRNA and depleting structured dsRNA contaminants. In direct comparisons, HPLC and cellulose chromatography yield similar reductions in dsRNA (as judged by J2 antibody signal) and comparably enhance protein expression *in vivo*, though both can leave trace amounts of immunostimulatory dsRNA. dsRNA-specific affinity chromatography, employing ligands that bind dsRNA with high selectivity, can achieve even greater reductions in dsRNA with less loss of single-stranded RNA (ssRNA) than either cellulose or reverse-phase methods[1].

Enzymatic purification provides an alternative approach to chromatographic separation methods. Controlled digestion of dsRNA in specific buffered conditions using RNase III can selectively degrade dsRNA with minimal loss of ssRNA. RNase III purification can also be implemented with standard laboratory equipment and minimal expertise.

1. Clark N, Kozarski M, Asci S..., **Removal of dsRNA byproducts using affinity chromatography,** Molecular Therapy Nucleic Acids, 2025; 36

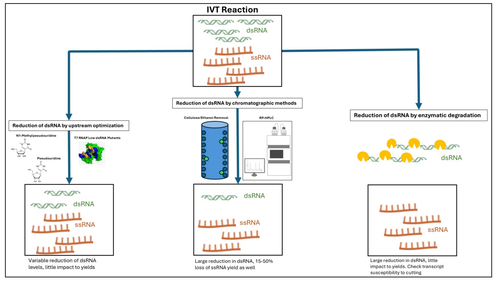

Figure 2. Primary methods of dsRNA removal by either protocol modification, chromatographic methods or enzymatic degradation.

Nucleotide Substitution and dsRNA Removal

We evaluated an enzymatic dsRNA removal strategy using the CELLSCRIPT™ Min-Immune™ Gold dsRNA Removal Kit to generate ultra low-mmunogenicity mRNA suitable for highly sensitive cellular applications. mRNAs were synthesized using uridine, pseudouridine or N1-methyl-pseudouridine, and then a subset of each mRNA was enzymatically treated. Quality analysis demonstrated that enzymatic treatment achieved a substantial reduction in dsRNA content while maintaining transcript integrity. Cell lines transfected with the mRNA constructs were assayed for luciferase activity at 18- and 48-hours post transfection. RT qPCR analysis was performed on total cellular RNA isolates from each cell line and mRNA construct to determine gene expression levels of immune sensors (TLR3, RIG I, MDA5, IFNA13, IFNB1, TNF, IL6, OAS1 and PKR).

Methods and Materials

mRNA Synthesis and Modification

RNA encoding NanoLuc® luciferase was synthesized using the INCOGNITO™ T7 mScript™ Complete mRNA Production System (CELLSCRIPT™, Madison, WI, USA). Three parallel IVT reactions containing either canonical uridine-5′-triphosphate (UTP), pseudouridine-5′-triphosphate (ΨTP), or N1-methyl-pseudouridine-5′-triphosphate (N1meΨTP) were transcribed from linearized DNA templates at 37°C for 30 min. IVT products were purified by NH_4OAc precipitation, washed with 70% ethanol, resuspended in RNase-free water and quantified by spectrophotometry.

The 5′ cap, Cap-1 modification, and poly(A) tail were added enzymatically using the capping and tailing modules included in the INCOGNITO™ T7 mScript™ Complete kit. Double-stranded RNA contamination was removed enzymatically using the Min-Immune™ Gold dsRNA Removal module included in the INCOGNITO™ T7 mScript™ Complete kit. The final NanoLuc mRNA was approximately 960 nt in length, including the poly(A) tail.

Enzymatic dsRNA Removal and Quality Assessment

mRNA quality was confirmed by capping efficiency and poly(A)-tail length QC assays (EZ QC™ kits, CELLSCRIPT™; procedures per manufacturer's instructions).

Table 1. Results from CELLSCRIPT™s EZ-QC™ Assay testing following the procedures in the Capping Efficiency, Cap 1 Efficiency, and Poly(A)-Tail Length Assay Kits.

Nucleotide	Average Poly(A)-Tail Length	Capping Efficiency	Cap 1 Efficiency
UTP	194-A's	100%	97.4%
ΨTP	188-A's	100%	95.1%
N1meΨTP	184-A's	100%	98.5%

dsRNA Quantification

Residual dsRNA was quantified by slot blot immunoassay using a dsRNA standard curve and the dsRNA-specific J2 monoclonal antibody (SCICONS).

Standards (50–250 pg dsRNA) and samples were applied to nitrocellulose in triplicate. For each condition, 1 µg of Min-Immune–treated mRNA and 100 ng of untreated mRNA were loaded, with additional dilution of untreated samples as needed to stay within the linear range of standard curve. Membranes were air dried, blocked in 5% (w/v) skim milk in TBS T for 1 hr, incubated overnight with J2 antibody (1 µg/mL in blocking buffer), washed and then incubated for 1 hr with HRP conjugated anti-mouse IgG (1:1000). After washing, signal was detected by ECL on a Syngene® G:Box imaging system. Slot intensities were fit to the standard curve by linear regression to determine dsRNA content before and after treatment.

Table 2. Results from Slot blot testing show a significant reduction in double stranded RNA contamination.

dsRNA Condition	Nucleotide	% dsRNA
Untreated	UTP	0.425%
	ΨTP	0.106%
	N1meΨTP	0.054%
Min-Immune™ Gold treated	UTP	0.000%
	ΨTP	0.005%
	N1meΨTP	0.002%
% Reduction of dsRNA after Min-Immune™ Gold treatment	UTP	99.91%
	ΨTP	95.66%
	N1meΨTP	96.65%

Transfection and NanoLuc Reporter Assay

NanoLuc mRNAs were transfected in duplicate into HEK293, A549 and JAWSII cells. Cells were maintained in serum-containing medium and seeded at 4 × 10^5 cells per well 24 hr before transfection. HEK293 and A549 cells were transfected with 500 ng mRNA per well using 1.25 µL (HEK293) or 3 µL (A549) Lipofectamine™ MessengerMax™ (Thermo Fisher Scientific) in 100 µL Opti MEM™-I medium. JAWSII cells were transfected with 250 ng mRNA and 1 µL transfection reagent under the same conditions.

Cells were incubated at 37°C with 5% CO_2 and harvested at 18 hours and 42 hours post transfection. After washing, cells were lysed in Reporter Lysis Buffer (Promega). Lysates were mixed 1:1 with Nano Glo Luciferase Assay Reagent (Promega) and relative light units (RLUs) were measured. Average RLUs from duplicate wells and duplicate assays were plotted for each condition.

Transfection and RT qPCR for Innate Immune Response

Human THP-1 Cells were transfected in duplicate with 1.5 µg of each mRNA using 2.5 µL transfection reagent per well. After 18 or 48 hours at 37°C, cells were collected, washed and total cellular RNA was isolated using the MasterPure™ Complete DNA and RNA Purification Kit (Biosearch Technologies).

Equal amounts of RNA from each sample were analyzed by one-step reverse transcription quantitative PCR (RT qPCR; iTaq Universal SYBR® Green One Step Kit, Bio-Rad) on a Bio-Rad CFX96 real time thermal cycler using primer sets for Toll like receptor 3 (TLR3), retinoic acid inducible gene I (RIG I), melanoma differentiation associated protein 5 (MDA5), interferon alpha 13 (IFNA13), interferon beta 1 (IFNB1), tumor necrosis factor (TNF), interleukin 6 (IL6), 2′-5′ oligoadenylate synthetase 1 (OAS1), protein kinase R (PKR) and glyceraldehyde 3 phosphate dehydrogenase (GAPDH). Quantification cycle (Cq) values were normalized to GAPDH and relative expression (ΔΔCq method) was calculated versus mock transfected controls and plotted for each target gene.

Results

Luciferase Expression

Nucleotide composition significantly affected NanoLuc mRNA performance. ΨTP and N1meΨTP mRNAs consistently outperformed canonical UTP across all cell types, timepoints, and treatment conditions. Min-Immune™ Gold treatment reduced these discrepancies but the trend was maintained, with ΨTP and N1meΨTP typically showing order-of-magnitude improvements over canonical UTP.

Min-Immune™ Gold treatment increased luciferase expression for all nucleotide types across both timepoints. In HEK293 cells, non-significant gains were observed at 18 hr for ΨTP and N1meΨTP (2.8% and 6.9%, respectively), with modest gains at 42 h (21% and 19%). The largest increases occurred with UTP mRNA (181–5470% depending on cell type/timepoint). Significant gains were also seen for ΨTP and N1meΨTP in A549 and JAWSII cells (33–355%), though N1meΨTP consistently showed smaller relative increases than ΨTP. In a cross-comparison between dsRNA removal and nucleotide type, UTP mRNA treated with Min-Immune™ Gold system outperformed untreated ΨTP and N1meΨTP in A549 cells (both timepoints) and JAWSII cells (ΨTP at 18 hr).

Table 3. Percent increase in expression of Min-Immune™ Gold treated mRNA compared to untreated mRNA.

Timepoint	Nucleotide	Cell Types		
		HEK293	A549	JAWSII
18-hour	UTP	+181%	+2004%	+2568%
	ΨTP	+3%	+102%	+226%
	N1meΨTP	+7%	+33%	+45%
42-hour	UTP	+792%	+5470%	+2671%
	ΨTP	+21%	+355%	+265%
	N1meΨTP	+19%	+78%	+45%

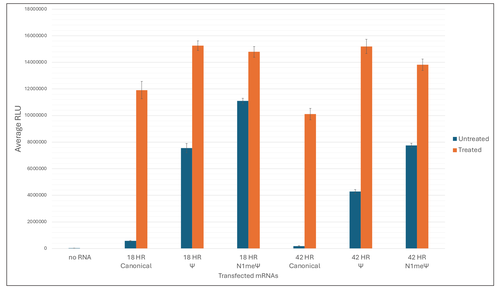

Figure 3. A549 cells assayed for luciferase activity at 18- and 42-hours post-transfection.

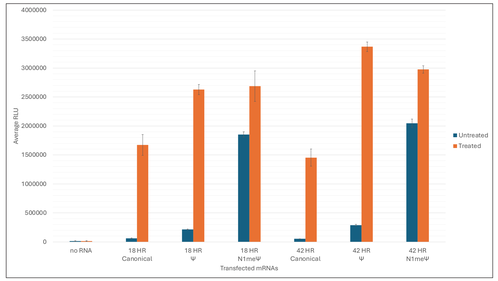

Figure 4. JAWSII cells assayed for luciferase activity 18- and 42-hours post-transfection.

Innate Immune Response

Nucleotide composition of mRNAs had a significant impact on immune stimulation, specifically the use of N1meΨTP in mRNA production as it provided the lowest untreated sample expression for sensor genes in every category.

Table 4. Normalized expression of Immune Sensors Genes for untreated samples of canonical UTP and % change in expression of untreated samples with ΨTP and N1meΨTP nucleotide substitutions

Gene	Expression ($\Delta\Delta C_t$) of Canonical Nucleotides (UTP)	Ψ vs UTP	N1meΨ vs UTP
IFNA13	7.58	-57%	-87%
TLR3	7.73	+4%	-79%
TNF	2.09	+14%	-56%
RIG-I	3.96	-10%	-61%
OAS1	5.91	-39%	-86%
MDA5	5.34	+28%	-80%
PKR	2.54	-5%	-68%
NOD2	6.03	-15%	-75%

Untreated mRNAs exhibited more immune stimulation than treated mRNAs in nearly all categories (IL6 expression of untreated N1meΨTP mRNA was lower than all other conditions, and IFNA13 expression of untreated N1meΨTP mRNA was lower than treated ΨTP mRNA). Average reduction of treating any nucleotide type (UTP, ΨTP or N1meΨTP) with Min-Immune™ Gold corresponded to a reduction in TLR3, RIG1, OAS1 and MDA5 expression of 81%, 86%, 86%, 83% respectively. TLR3, RIG1, OAS1 and MDA5 are primary dsRNA sensors and would be predicted to be very strongly influenced by small amounts of dsRNA. It is also shown that RIG1 was strongly influenced by as little as 0.0537% dsRNA remaining in untreated N1meΨTP mRNA, despite N1meΨTP showing a good reduction in immunogenicity in many other categories when compared to UTP.

IFNB1 and IL6 expression (presented as separate graphs due to much larger spread of data) was significantly lower for untreated N1meΨTP compared to untreated UTP or ΨTP. Treatment with the Min-Immune™ Gold system appears to reduce both UTP and ΨTP down to a comparable level as N1meΨTP, largely evening out results.

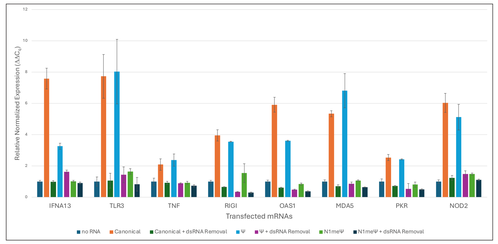

Figure 5. Relative normalized expression of immune sensor genes.

Table 5. Average expression of immune sensor genes for untreated and treated samples for all nucleotides, grouped by gene.

Gene	Untreated Average Expression Expression ($\Delta\Delta C_t$)	Treated Average Expression Expression ($\Delta\Delta C_t$)	% Reduction
RIG-I	3.02	0.43	–86%
PKR	1.92	0.59	–69%
OAS1	3.46	0.49	–86%
TLR3	5.80	1.11	–81%
NOD2	4.21	1.28	–70%
MDA5	4.41	0.74	–83%
TNF	1.80	0.85	–53%
IFNA13	3.96	1.18	–70%

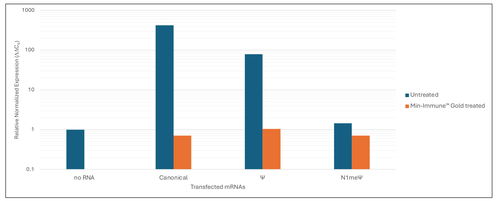

Figure 6. Relative normalized expression of IFNB1 for each mRNA type.

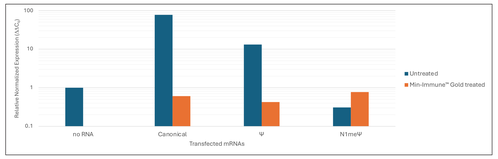

Figure 7. Relative normalized expression of IL6 for each mRNA type.

Conclusion

Enzymatic degradation of dsRNA contaminants dramatically enhances mRNA therapeutic performance across all nucleotide compositions while potently suppressing innate immune activation. Luciferase expression data revealed approximately 1.8–55-fold improvements for canonical UTP mRNA in HEK293, A549 and JAWSII cells, with 1.3–4.6-fold improvements for ΨTP/N1meΨTP transcripts. UTP mRNA with dsRNA removal outperformed untreated nucleotide substituted mRNAs in immune-sensitive A549 (both timepoints) and JAWSII (ΨTP at 18 hr) cells, while reducing expression decay for UTP transcripts or expression gains for Ψ or N1meΨ transcripts from 18-hour to 42-hour timepoints.

qPCR analysis of 8 innate immune sensors confirmed and an average of 4-5-fold reduction in immune activation, normalizing all nucleotide types to near baseline (no-RNA control). dsRNA-specific sensors (RIG-I, TLR3, MDA5 and OAS1) showed particularly strong suppression (>5-fold decrease), with treated conditions outperforming untreated N1meΨ by roughly 1.5-1.8-fold across genes. N1meΨ mRNA benefited from a further ~1.8-fold reduction in immune sensor pathways when treated for dsRNA.

Benefits of enzymatic dsRNA degradation:

- Universal rescue of canonical UTP to closely match nucleotide substituted mRNA performance
- Additive to nucleotide substitution (N1meΨ gained ~1.8-fold more suppression of immunogenicity)
- Expression stability (reduce or eliminate 18–42 hr decay seen in untreated samples)
- Targeted dsRNA sensor suppression for clinical-grade mRNA/LNP applications

These findings establish enzymatic dsRNA removal as an essential orthogonal purification for next-generation mRNA therapeutics, enabling high expression with minimal immunogenicity across diverse delivery contexts.

RNase III can potentially cleave structured or partially duplexed regions within the intended transcript and the extent of clipping should be empirically assessed for each mRNA.