# Enzymatic Purification Strategies to Eliminate dsRNA: Advancing Translational Efficiency and Immune Compatibility of IVT-mRNA

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## Introduction

Messenger RNA (mRNA) and self-amplifying RNA (saRNA) 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 protein expression and compromise cellular viability.

## Effects of dsRNA and Importance of dsRNA Removal

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 RIGI, 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 RIGI can still be activated at dsRNA levels on the order of ~0.05% (%dsRNA of total mRNA in sample) in a single dose of N1mΨ-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.

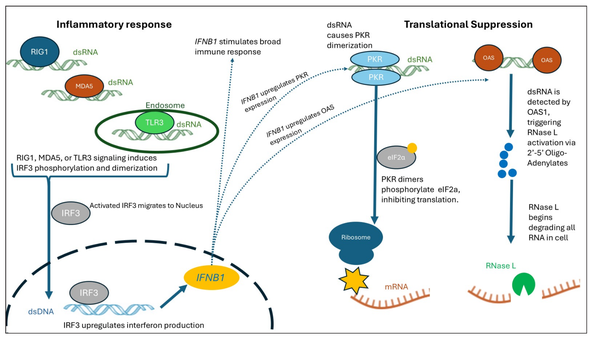


**Figure 1.** Inflammatory response and translation suppression mechanisms activated by dsRNA.

## Purification Methods for dsRNA Removal

Generated 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 therapuetic 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.[1] 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.

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.

## 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-immunogenicity mRNA suitable for highly sensitive cellular applications. mRNAs encoding luciferase 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 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, RIGI, MDA5, IFNA13, IFNB1, TNF, IL6, OAS1 and PKR).

## Self-amplifying RNA

saRNAs are engineered derivatives of positive-sense single-stranded RNA viruses in which viral structural protein-coding sequences are replaced with a gene of interest, while nonstructural proteins required for RNA replication, including the viral RNA-dependent RNA polymerase (RdRP), are retained. Following delivery, saRNAs undergo intracellular replication, amplifying the RNA template and enabling sustained transgene expression from low input doses.[2] These properties have established saRNAs as a promising platform for vaccines and gene-based therapeutics. A major limitation of saRNA systems is activation of host innate immune responses. Replication generates dsRNA intermediates and other pathogen-associated molecular patterns that are recognized by pattern recognition receptors, including Toll-like receptors (TLR3, TLR7 and TLR8), MDA5, RIGI, PKR, and the OAS pathway.[3] Activation of these pathways induces Type I interferon (IFN) signaling and antiviral programs that reduce RNA stability and translation, limiting transgene expression.[3,4] While beneficial for vaccines, excessive IFN responses are detrimental to saRNA performance. While incorporation of modified nucleotides (e.g., pseudouridine or N1-methyl-pseudouridine) can mitigate innate immune activation and improve translation in conventional mRNA systems, such substitutions are generally not well tolerated or as effective in saRNA due to the substrate requirements of the viral RdRP, which relies on canonical nucleotides for efficient replication. As a result, alternative strategies are required to reduce immunogenicity in saRNA.

One such approach is the selective removal of dsRNA contaminants generated during *in vitro* transcription, which represents a major trigger of innate immune activation. By reducing dsRNA content without altering nucleotide composition, it is possible to attenuate IFN responses while preserving replicative competence. This strategy offers a means to improve saRNA translation and overall functional output without compromising the amplification mechanism that underlies its potency.

The effect of dsRNA removal on saRNA was evaluated using the Min-Immune™ Gold purification module. A 9-kb capped and polyadenylated saRNA encoding green fluorescent protein (GFP) served as the model construct. RNA integrity was assessed by denaturing agarose gel electrophoresis, and functional output was quantified by GFP expression following transfection into cultured cells. Activation of dsRNA-responsive innate immune pathways was measured by RT-qPCR. These studies define how dsRNA depletion improves saRNA expression while reducing innate immune activation.

## Methods and Materials

### Transfection and NanoLuc Reporter Assay

NanoLuc mRNAs were transfected in duplicate into HEK 293, A549 and JAWSII cells. Cells were maintained in serum-containing medium and seeded at 4 × $10^5$ cells per well 24 hr before transfection. HEK 293 and A549 cells were transfected with 500 ng mRNA per well using 1.25 µL (HEK 293) or 3 µL (A549) Lipofectamine™ MessengerMax™ (Thermo Fisher Scientific) in 100 µL Opti MEM™. 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 (RIGI), 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.

### saRNA Template and *In Vitro* Transcription

A plasmid encoding a Venezuelan equine encephalitis (VEE) virus-based saRNA expressing green fluorescent protein (GFP) (T7-VEE-GFP; NovoPro Bioscience, Cat. #V016969)[5] was used as the transcription template. The 11.5-kb plasmid was linearized with Mlu I (New England Biolabs) and purified by phenol-chloroform extraction followed by salt/EtOH precipitation. Linearized plasmid was used as template for *in vitro* transcription with the T7 mScript™ Complete Standard mRNA Production System (CELLSCRIPT™) according to the manufacturer's protocol, yielding a 9,696-nt saRNA containing a Cap 1 structure and poly(A) tail. dsRNA contaminants were removed using the Min-Immune™ Gold dsRNA Removal module of T7 mScript Complete Standard mRNA Production system according to the manufacturer's instructions and analyzed to confirm integrity.

### saRNA and Innate Immune Activation Assay

THP-1 cells were used to assess activation of dsRNA-responsive innate immune pathways. Cells were transfected with 2 µg saRNA using 3.5 µL Lipofectamine™ MessengerMAX™. At 28-hr post-transfection, cells were harvested, washed and total RNA was isolated using the MasterPure™ Complete DNA and RNA Purification Kit (Biosearch Technologies). For gene expression analysis, 25 ng RNA per reaction was analyzed by one-step RT-qPCR using the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad) on a CFX96 real-time PCR system (Bio-Rad). Twelve primer sets targeting dsRNA-responsive genes were used. Reactions were performed in technical replicates. Gene expression was normalized to GAPDH and calculated relative to mock-transfected controls using the ΔΔCq method.

## 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 hr (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 1:** Percent increase in luciferase activity of Min-Immune Gold™ treated mRNA compared to untreated mRNA.

| | | Cell Types | | |
|---|---|---|---|---|
| **Timepoint** | **Nucleotide** | **HEK293** | **A549** | **JAWSII** |
| 18-hour | UTP | + 181% | + 2,004% | + 2,568% |
| | ΨTP | + 3% | + 102% | + 226% |
| | N1meΨTP | + 7% | + 33% | + 45% |
| 42-hour | UTP | + 792% | + 5,470% | + 2671% |
| | ΨTP | + 21% | + 355% | + 265% |
| | N1meΨTP | + 19% | + 78% | + 45% |

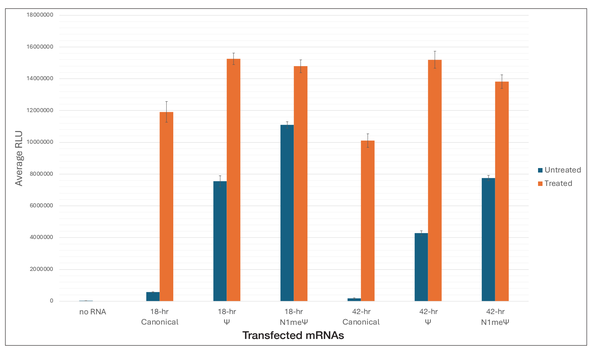


**Figure 2:** A549 cells assayed for luciferase activity at 18- and 42-hours post-transfection.

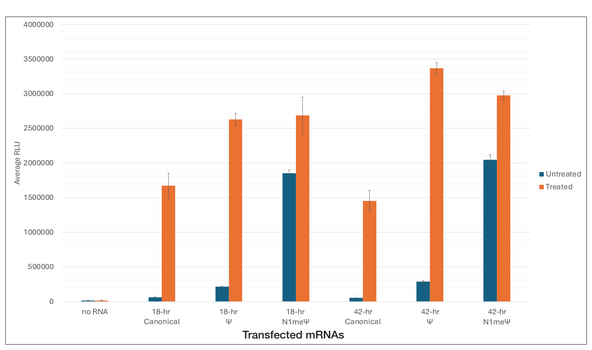


**Figure 3:** JAWSII cells assayed for luciferase activity 18- and 42-hours post-transfection.

## 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.

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.

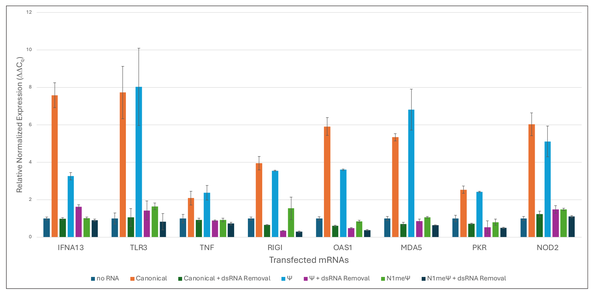


**Figure 4:** Relative normalized expression of immune sensor genes.

**Table 2:** Average expression of immune sensor genes for untreated and treated samples for all nucleotides, grouped by gene.

| Gene | Untreated Average Expression Expression (ΔΔCq) | Treated Average Expression Expression (ΔΔCq) | % Reduction |
|---|---|---|---|
| RIGI | 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% |

THP-1 monocytes were transfected with VEE-GFP saRNA that was either untreated or subjected to 15- or 60-minute dsRNA removal prior to transfection. This experiment assessed whether dsRNA depletion improves saRNA performance in an immune-sensitive cell type. Untreated saRNA produced minimal GFP expression, consistent with strong innate immune activation. In contrast, dsRNA-depleted samples showed a marked increase in GFP signal at both timepoints, indicating improved functional output. Similar results between 15- and 60-minute treatments suggest rapid and effective dsRNA removal. These data demonstrate that dsRNA contaminants limit saRNA expression in THP-1 cells, and that their removal restores GFP expression, supporting dsRNA depletion as a key strategy to enhance saRNA efficacy.

**Figure 5:** saRNA Fluorescence images of THP-1 monocytes 28-hr post transfection with VEE-GFP dsRNA. **dsRNA removal enhances saRNA mediated GFP expression in THP-1 monocytes.**

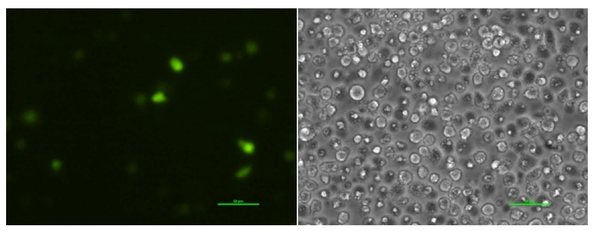

**Figure 5A:** THP-1 monocytes transfected with untreated VEE-GFP saRNA show very weak GFP expression at 28-hr post transfection.

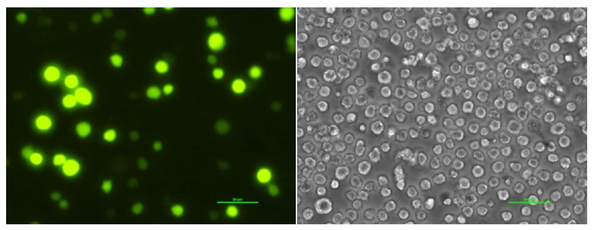

**Figure 5B:** THP-1 monocytes transfected with VEE-GFP saRNA subjected to a 60-minute dsRNA removal treatment exhibit increased GFP expression with preserved cell morphology.

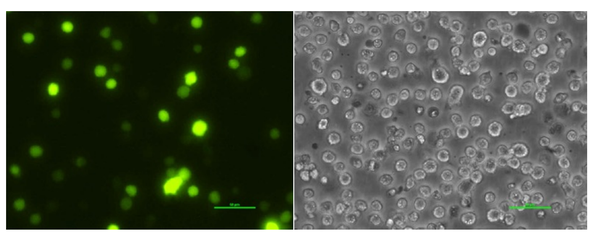

**Figure 5C:** THP-1 monocytes transfected with VEE-GFP saRNA subjected to a 15-minute treatment also show enhanced GFP expression compared to untreated saRNA, indicating that short dsRNA removal treatment is sufficient.

## RT-qPCR

Total RNA was harvested post imaging, and expression of dsRNA sensors and innate immune response genes was quantified by RT-qPCR. Bars represent relative normalized expression (ΔΔCq) of **RIGI, MDA5, OAS1, TLR3, TLR7, TLR8, TNF, PKR and NLRP3**, compared to mock transfected controls. Untreated VEE-GFP RNA induced strong upregulation of TLR3, TLR7, TLR8 and TNF, with TNF showing the highest induction. In contrast, dsRNA removal substantially reduced innate immune activation, with both 15-minute and 60-minute treatments returning expression of most sensors and cytokines to levels comparable to mock. Data shown as mean ± SD.

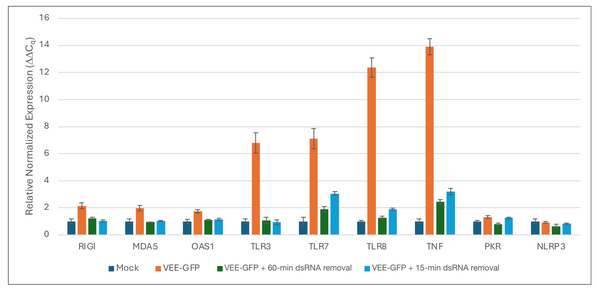


**Figure 6A.** RT-qPCR analysis of innate immune gene expression in THP-1 cells following VEE-GFP transfection with or without dsRNA removal.

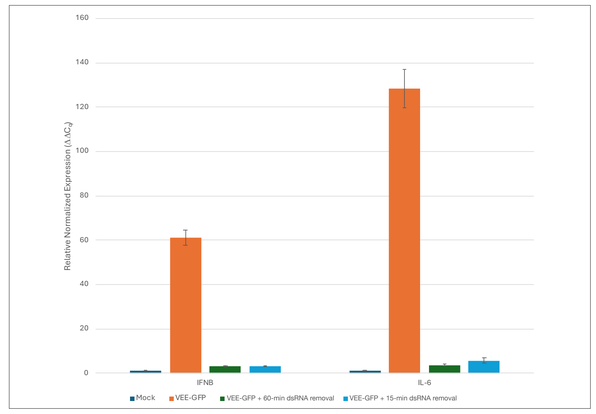


**Figure 6B.** RT-qPCR analysis of IFNB and IL-6 expression following VEE-GFP transfection with or without dsRNA removal.

## Conclusion

Enzymatic degradation of dsRNA contaminants markedly improves RNA therapeutic performance by simultaneously enhancing expression and suppressing innate immune activation across both mRNA and saRNA platforms. dsRNA removal increased luciferase expression by ~1.8–55-fold for canonical UTP mRNA and 1.3–4.6-fold for Ψ/N1mΨ-modified transcripts across multiple cell types, while stabilizing expression over time. Notably, dsRNA-depleted UTP mRNA outperformed untreated nucleotide-modified mRNAs in immune-sensitive models. RT-qPCR analysis demonstrated a ~4–5-fold reduction in innate immune activation, with pronounced suppression (>5-fold) of dsRNA-specific sensors (RIGI, TLR3, MDA5, OAS1), and additional immunogenicity reductions observed even for N1mΨ-modified transcripts.

Consistent with these findings, dsRNA contaminants were identified as the primary drivers of innate immune activation and reduced functional output in VEE-GFP saRNA. In THP-1 monocytes, untreated saRNA induced strong cytokine and dsRNA sensor activation with minimal protein expression, whereas dsRNA removal restored immune signaling to near-baseline levels and rescued expression. These improvements were achieved rapidly (within 15–60 minutes) and translated to enhanced GFP expression and preserved viability in HEK293 cells. Despite minor transcript clipping, the overall gains in expression, immune compatibility and functional performance establish enzymatic dsRNA removal as a critical, orthogonal purification strategy for next-generation RNA therapeutics.

## Key Findings

- Enzymatic degradation of dsRNA contaminants dramatically enhances mRNA therapeutic performance across all nucleotide compositions.
- Untreated VEE-GFP saRNA induced strong upregulation of dsRNA sensors and cytokines in THP-1 cells.
- IFNB and IL-6 hyper-induction was nearly eliminated by dsRNA removal.
- Both 15- and 60-minute dsRNA-removal treatments sharply reduced innate immune gene expression to near-baseline.
- dsRNA impurities are the primary drivers of saRNA-triggered innate immune activation.

## References:

1. Clark N. *et al.* (2025) Molecular Therapy Nucleic Acids 36(2).
2. Blakney A. *et al.* (2021) Vaccines (Basel) 9(2),97.
3. Minnaert A.K. *et al.* (2021) Advanced Drug Delivery Reviews 176, 113900.
4. De Haro C. *et al.* (1996) FASEB Journal 10:1378.
5. Yoshioka, N. *et al.* (2013) Cell Stem Cell 13(2), 246.

**Additional Information**

Immunogenicity Reduction

Min-Immune™ Gold

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