Strengthening Control of Double-Stranded RNA in mRNA Manufacturing Processes

by Senne Dillen

Senior Scientist Drug Substance Process Development

A major challenge in mRNA production is reducing dsRNA contamination. In order to address this challenge, etherna has developed an optimized mRNA manufacturing process. This optimized process decreases dsRNA levels in a way that is compatible with large scale GMP-grade manufacturing and results in ultra-pure mRNA that avoids activation of the innate immune system.

Detection of double-stranded RNA by endosomal Toll-Like-receptors (TLR) or cytoplasmic RIG-I-like receptors leads to the expression of proinflammatory cytokines and type I interferons, resulting in the induction of an inflammatory response and inhibition of protein expression [1]. As such, for an mRNA therapeutic to reach maximal efficacy, it is critical that dsRNA content is well-controlled during the manufacturing process.

Elimination of dsRNA in downstream manufacturing processes is routinely performed using two main methods: cellulose-based purification and reversed-phase HPLC.

Cellulose-based purification has been extensively described in the past as a tool to purify viral double-stranded RNA from plant and fungal tissues [2-4]. More recently, similar purification processes have been described aimed at the purification of dsRNA from in vitro transcribed RNA [5]. Large amounts of cellulose slurry are, however, required to process limited amounts of RNA. The chemical compatibility of the crude cellulose fibers with the caustic agents required for in-process sanitization is also poor, limiting the method's scalability and preventing its use in large scale manufacturing processes used for the production of clinical or commercial grade mRNA products [6].

The removal of dsRNA impurities in the downstream manufacturing process can also be accomplished using ion-pair reversed phase (IP-RP) HPLC, as described by Weissman et al. [7]. While this process potently reduces dsRNA levels, the scalability of this process is significantly limited by the use of toxic or flammable organic solvents, the requirement of working at elevated temperature and the very limited dynamic binding capacity of most IR-IP resins.

The effect of cellulose purification and IP-RP chromatography on dsRNA levels and mRNA efficacy appears to be similar, with both leading to similarly sized reduction in dsRNA content and increased vivo efficacy [5]. Activation of an innate immune response can however not be fully prevented by only using these downstream processes. Both Baiersdörfer et al. and Nelson et al. demonstrate that innate activation can only be abolished using a combination of a downstream process aimed at dsRNA removal and the use of N1-methylpseudouridine during in vitro transcription [5, 8].

A Paradigm Shift in mRNA Manufacturing

The approach of **etherna** differs from those posited by Baiersdörfer et al. and Nelson et al. in the sense that control of dsRNA content begins in the upstream process. By initially focusing on the optimization of the in vitro transcription reaction, **etherna's** manufacturing processes generate mRNA characterized by high capping efficiency and exceptionally low dsRNA content, even when using unmodified NTPs (Figure 1).

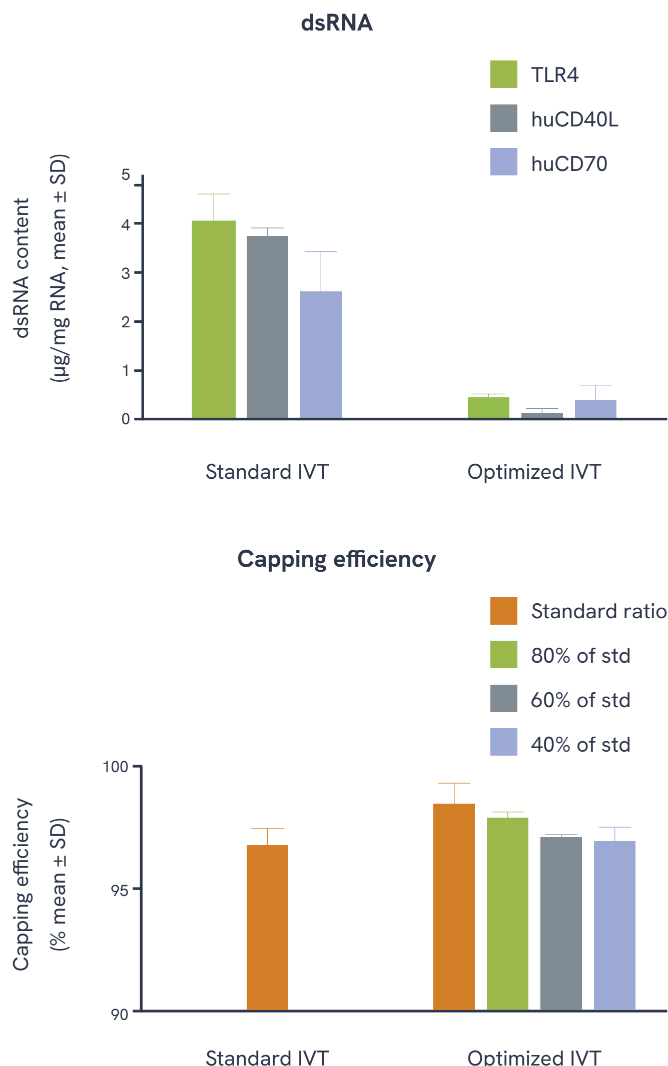


Figure 1
Top: dsRNA content of three RNA constructs produced using standard and optimized IVT processes (unmodified NTPs).
Bottom: capping efficiency of TLR4 mRNA produced using standard IVT and optimized IVT using 4 levels of cap analog.

Similar to the observations described by Baiersdörfer et al. and Nelson et al., m1Ψ modified eGFP mRNA that was not subjected to additional purification aimed at the reduction of dsRNA levels, showed significantly lower in vitro expression compared to the same material that was subjected to cellulose purification. In contrast, eGFP mRNA produced using the optimized IVT process and subsequent silica purification showed similarly high expression levels as mRNA produced using the standard IVT process followed by cellulose purification (Figure 2).

Evaluation of innate immune activation using the A549-Dual reporter cell line demonstrated a significant reduction in the activity of the IFN regulatory pathway due to cellulose purification of the mRNA produced using the standard IVT process. Remarkably, silica-purified mRNA produced using the optimized process showed no signs of innate activation. (Figure 2).

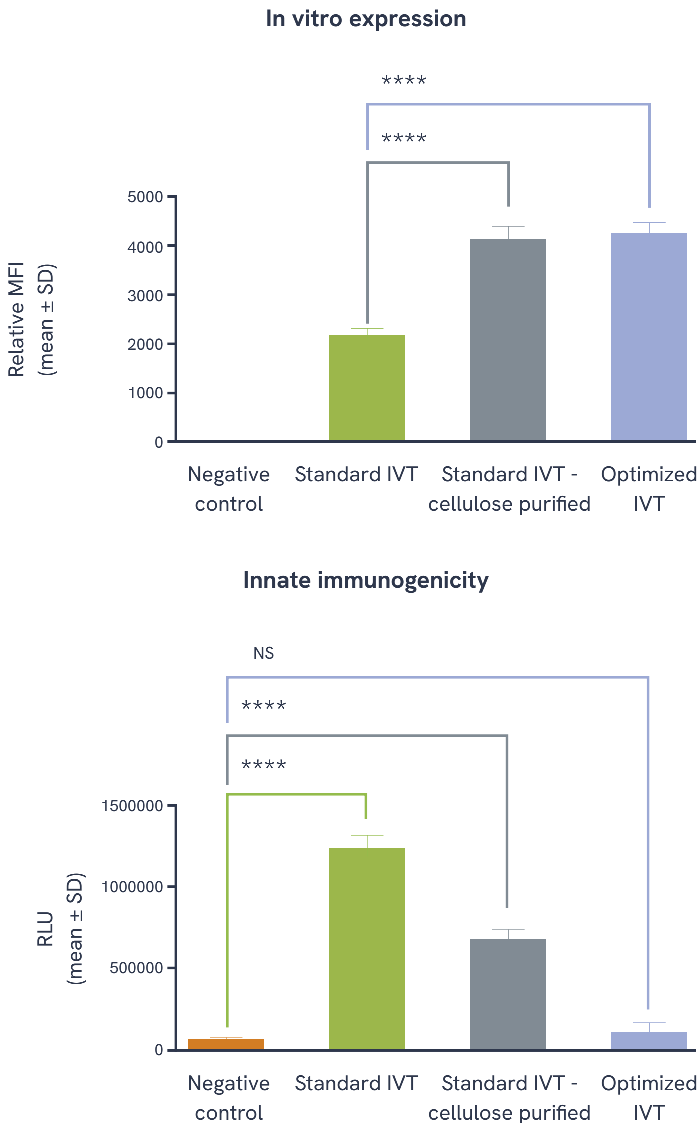
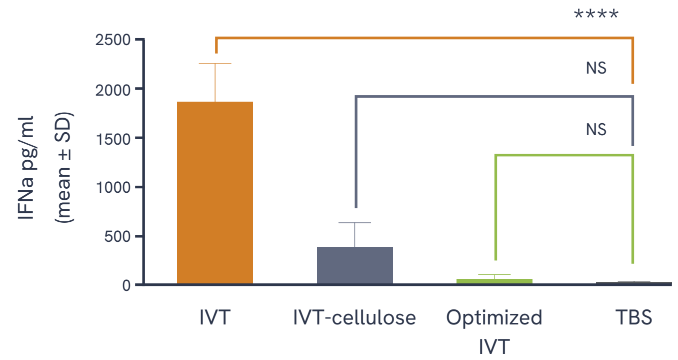


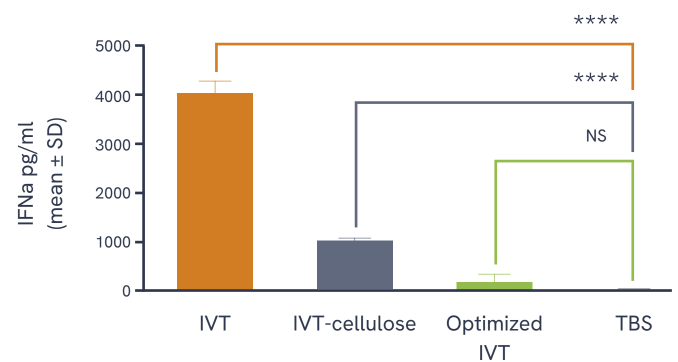
Figure 2

Top: *In vitro expression of m1Ψ modified eGFP mRNA produced using either standard or optimized IVT and purified using silica spin column with and without additional cellulose purification step.*

Bottom: *Innate immunogenicity as determined using A549-dual reporter cell line.*

The observations described in Figure 2 were confirmed in vivo where cellulose purification of mRNA produced using the standard IVT process lead to a significant decrease in IFN-α titers 6 hours after prime or boost injection. Most notably, animals injected with LNPs encapsulating mRNA produced using the optimized IVT process did not shown increased IFN-α titers compared to animals injected with saline solution (Figure 3).





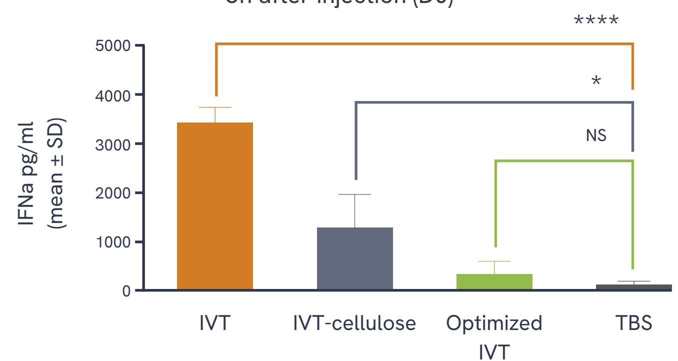


Figure 3

IFN-α titers of mice injected with LNPs encapsulating m1Ψ modified SARS-COV-2 mRNA or luciferase mRNA produced using either standard or optimized IVT and purified using silica spin column with and without additional cellulose purification step.

Reducing dsRNA levels using etherna's optimized in vitro transcription reaction resulted in the production of ultra-pure mRNAwhich shows increased efficacy of mRNA expression in vitro and does not lead to activation of the innate immune system.

Highly Efficacious and Innate Immune Silent mRNA at Any Scale

Current best practices in mRNA manufacturing consist of using modified nucleotide triphosphates in combination with specialized purification steps aimed at the reduction of dsRNA levels. Only when these factors are combined is efficacy maximized and innate activation abolished. Scalability of the applied purification processes is however limited due to low binding capacities, use of flammable or toxic solvents, low process yields and high temperature requirements.

etherna has developed a manufacturing process that demonstrates excellent control of dsRNA by minimizing dsRNA formation in the upstream process prior to further downstream purification. By optimizing the manufacturing process **etherna** is able to manufacture highly efficacious mRNA that shows no signs of innate immune activation from milligram to multi-gram scale.

> **etherna** has developed a manufacturing process that demonstrates excellent control of dsRNA by minimizing dsRNA formation in the upstream process prior to further downstream purification resulting in ultra-pure mRNA that avoids activation of the innate immune system.

References

1. Zhang, C., et al., Advances in mRNA Vaccines for Infectious Diseases. Frontiers in Immunology, 2019. 10.
2. hoi, Y.G. and J.W. Randles, Microgranular Cellulose Improves dsRNA Recovery from Plant Nucleic Acid Extracts. BioTechniques, 1997. 23(4): p. 610-611.
3. Bhat, A.I. and G.P. Rao, Isolation of Double-Stranded (ds) RNA from Virus-Infected Plants, in Characterization of Plant Viruses : Methods and Protocols, A.I. Bhat and G.P. Rao, Editors. 2020, Springer US: New York, NY. p. 299-302.
4. Okada, R., et al., A simple and rapid method to purify viral dsRNA from plant and fungal tissue. Journal of General Plant Pathology, 2015. 81(2): p. 103-107.
5. Baiersdörfer, M., et al., A Facile Method for the Removal of dsRNA Contaminant from In Vitro Transcribed mRNA. Molecular Therapy - Nucleic Acids, 2019. 15: p. 26-35.
6. Budtova, T. and P. Navard, Cellulose in NaOH-water based solvents: a review. Cellulose, 2016. 23(1): p. 5-55.
7. Weissman, D., et al., HPLC Purification of In Vitro Transcribed Long RNA, in Synthetic Messenger RNA and Cell Metabolism Modulation: Methods and Protocols, P.M. Rabinovich, Editor. 2013, Humana Press: Totowa, NJ. p. 43-54.
8. Nelson, J., et al., Impact of mRNA chemistry and manufacturing process on innate immune activation. Science Advances. 6(26): p. 6893.

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For more information feel free to reach out

+32 3 369 17 40
info@etherna.be
Galileilaan 19, 2845 Niel, Belgium

www.etherna.be