Two steps to ultra-low dsRNA: A GMP-ready path to safer RNA with engineered enzymes and dedicated purification

Introduction

Double-stranded RNA (dsRNA) is a known process-related byproduct of *in vitro* transcription (IVT) and a highly undesirable impurity in therapeutic RNA applications. Because dsRNA may resemble viral genomes, it is immunogenic by nature. It can activate the innate immune system via pattern recognition receptors, trigger the release of pro-inflammatory cytokines, and thereby cause unwanted side effects. The same immune response can also inhibit translation and thus reduce the efficacy of the therapeutic RNA.

To address this, WACKER developed a new process that systematically minimizes dsRNA across both upstream and downstream processing. By introducing an engineered T7 RNA polymerase and adding a dedicated dsRNA removal step, the approach reduces dsRNA to near undetectable levels. The study presented here was performed at WACKER's corporate R&D site.

Study aim

This study set out to establish a robust, scalable method for producing ultra-pure RNA with minimal immunogenic potential for advanced therapeutic applications. An important requirement was that the process remains compatible with Good Manufacturing Practice (GMP).

The process was designed around two complementary principles:

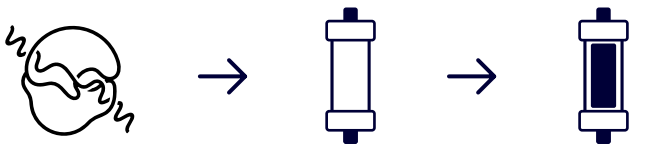
1. Preventing dsRNA formation during IVT with a novel engineered T7 RNA polymerase
2. Removing residual dsRNA during downstream processing (DSP) with a dedicated purification technology

Study method

Two process designs frame this study. A standard RNA production process runs IVT with wild-type T7 polymerase, followed by Oligo dT purification, enzymatic capping with Vaccinia Capping Enzyme and 5' methyltransferase, and a second Oligo dT purification. An optimized process consists of two changed elements: a T7 polymerase engineered to suppress dsRNA generation takes the place of the wild-type enzyme, and an additional purification step specifically captures dsRNA impurities (Figure 1).

For the proof-of-concept, the WACKER nucleic acid R&D team produced RNA with both the wild-type and the engineered T7 polymerase and took each IVT reaction through the standard production process described above but purified additionally with a specific dsRNA removal resin (Figure 1). The model construct was a standard 1.8 knt firefly luciferase (FLuc) mRNA carrying a 124-nucleotide poly(A) tail, made without nucleotide analogs. The batches from both polymerases were sampled for analytical characterization before the dsRNA removal step and after final purification. Sampling before and after dsRNA removal allowed the contribution of each process stage to be evaluated directly.

Figure 1: Low dsRNA process workflow



IVT
Generate RNA

Capture
Purify full-length RNA

Polishing
Remove dsRNA

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Study results

The results show that the two steps of the method make distinct and complementary contributions to minimizing dsRNA. The engineered T7 polymerase efficiently limits the formation of dsRNA during IVT. This reduced the dsRNA level after transcription from approximately 3.6% to 0.005% (Figure 2, left side). Although this reduction was substantial, it was accompanied by a ~60% reduction in IVT RNA titer (Figure 3).

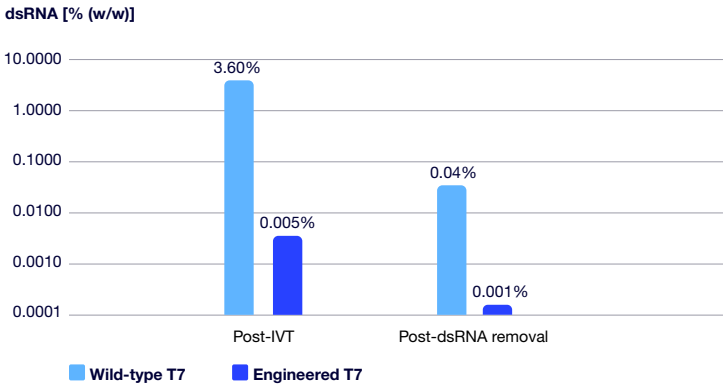


Figure 2: dsRNA content [%] after IVT (before dsRNA removal) and after dsRNA removal measured with Promega's Lumit® dsRNA detection assay.
(Note: The scale is logarithmic in order to see the low dsRNA values.)

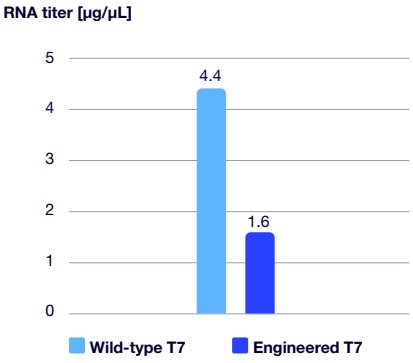


Figure 3: RNA yield [µg/µL] after IVT from different T7 polymerases.

The downstream purification step then removed residual dsRNA that remained after IVT. The dsRNA removal resin retained this impurity until the column reached saturation and reduced dsRNA content by up to 98.9%. For RNA produced with the wild-type T7 polymerase, dsRNA depletion purification lowered the dsRNA level from 3.6% to 0.04% (Figure 2, right side). When the same purification step was applied to mRNA produced with the engineered T7 polymerase with an initial dsRNA content of 0.005%, the final dsRNA concentration fell below 0.001%. The first step therefore minimizes dsRNA formation at its source, while the second removes the small residual fraction, resulting in final RNA substance with nearly undetectable dsRNA.

The improvement in RNA purity also resulted in improved cell culture results. In HEK293 cells, viability remained above 99% at 24 hours after transfection compared with untreated cells, showing that the cells were essentially unaffected by the purified RNA. At the same time, luciferase activity, used as the measure of RNA expression, was 32-45% higher than the commercial control RNA (Figure 4). This increase is particularly relevant because the purpose of a therapeutic RNA is to direct cells to produce the RNA-encoded protein without adding unnecessary stress on the cells. Higher expression means that more of the intended protein can be generated from the delivered RNA, strengthening the functional effect of the therapeutic. Together, the high cell viability and increased expression show that reducing dsRNA improves both the cellular tolerability of the RNA and its ability to perform its intended function.

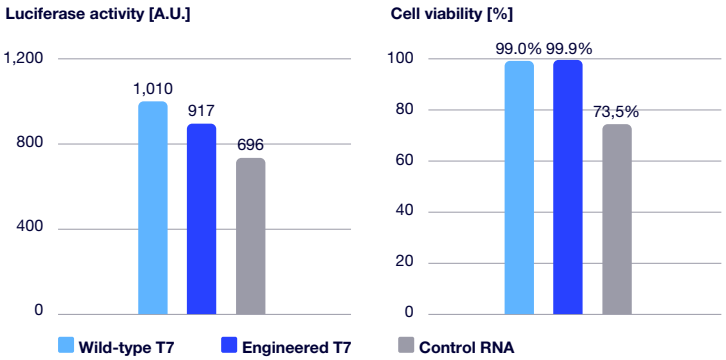


Figure 4: Luciferase activity [A.U.] in (left chart) and cell viability [%] of (right chart) HEK293 at 24 hours after transfection. The control RNA represents a commercially available firefly luciferase mRNA.

Taken together, the proof-of-concept demonstrates the value of addressing dsRNA at both stages of the process. The engineered T7 polymerase prevents most dsRNA from being formed, and the dedicated dsRNA resin removes residual impurity. This prevention and removal strategy lowered the final dsRNA concentration below 0.001%. By combining prevention with removal, the process produces high-quality RNA with ultra-low dsRNA levels that meet the expectations of nucleic acid therapeutics developers and regulators, while remaining suitable for scalable, GMP-ready manufacturing.

If you are interested in support for design of a low dsRNA process, engage with our process development services and experts by emailing us at **info.biologics@wacker.com**.

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