Double-stranded RNA (dsRNA) the byproduct of IVT

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Introduction

The approval of mRNA-based vaccines developed by Pfizer/BioNTech and Moderna, as authorized by the FDA to combat the global COVID-19 pandemic, has significantly advanced the field of mRNA therapeutics. These vaccines highlight the potential of mRNA technology due to their high potency, rapid development, cost-effective manufacturing, and safe administration[1]. As a result, mRNA-based approaches are now widely regarded as a strong alternative to conventional vaccine platforms.

In mRNA-based therapeutics, the synthesis of mRNA is primarily carried out through in vitro transcription (IVT) using T7 RNA polymerase. This method allows for efficient and scalable production of mRNA from a DNA template. However, previous studies have identified the formation of various byproducts during the transcription process[2,3]. Among these, double-stranded RNA (dsRNA) is particularly concerning, as it can trigger innate immune responses and substantially reduce the therapeutic efficacy of mRNA-based treatments.

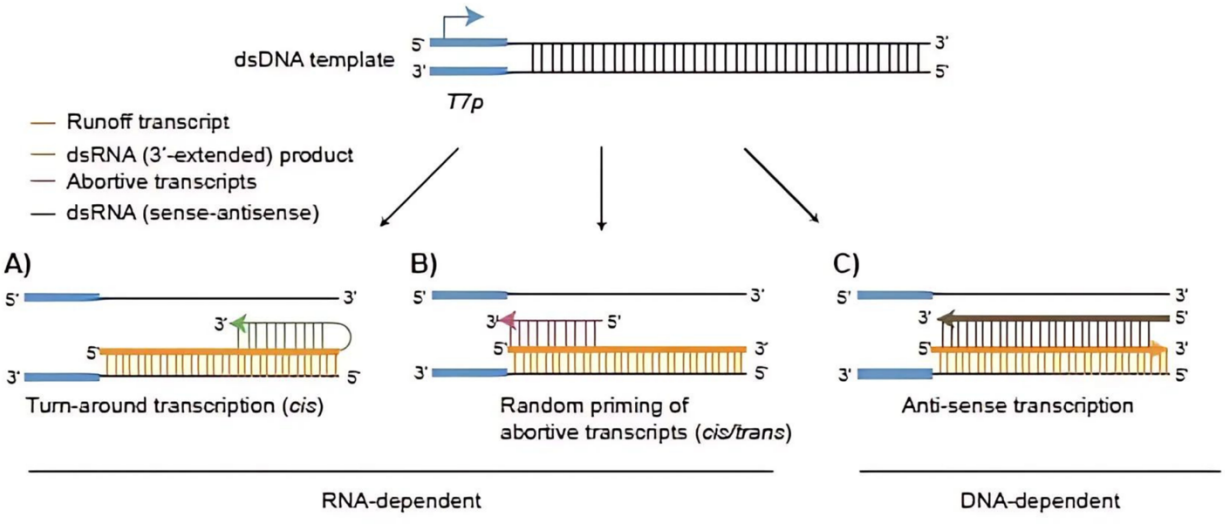

Figure 1. Schematic representation of possible mechanisms of dsRNA byproduct formation during in vitro transcription.[5]

Mechanisms of dsRNA Formation

The synthesis of dsRNA byproducts during in vitro transcription (IVT) can occur through two primary mechanisms[2,4,5] (Fig. 1.). One mechanism involves the runoff transcript produced by T7 RNA polymerase folding back on itself when the 3' end contains sufficient sequence complementarity, enabling self-priming and the formation of loop-back structured dsRNA. An alternative pathway involves T7 RNA polymerase transcribing from a promoter-less DNA strand, generating an RNA molecule complementary to the original runoff transcript. Additionally, short RNA fragments generated during transcription can anneal to complementary regions within the runoff transcript, further contributing to dsRNA formation. dsRNA is a major byproduct of IVT-based mRNA production and is notoriously difficult to eliminate completely.

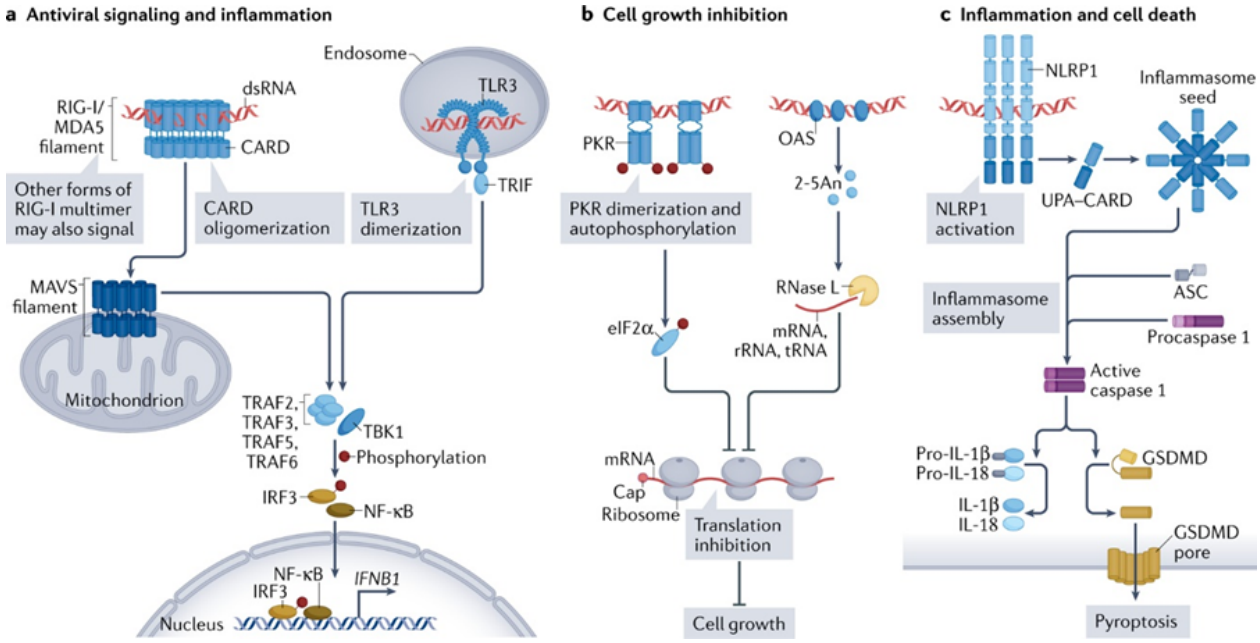

Figure 2. dsRNA sensors and their signaling.[7]

Immune-stimulating activity of dsRNA

dsRNA is recognized as a potent pathogen-associated molecular pattern (PAMP) and can activate several immune response pathways[1,3,6]. This activation occurs through pattern recognition receptors in various cellular compartments, leading to the production of type I interferon and other inflammatory mediators. Such responses are crucial as they can influence the overall efficacy of mRNA-based therapies by modulating immune system activity[7-9] (Fig. 2). Therefore, accurate quantification of dsRNA residues is key to ensuring mRNA drug substance quality.

Detection of dsRNA

One of the primary methods for detecting dsRNA byproducts in IVT reactions is the immunoblot assay. This approach utilizes dsRNA-specific antibodies to detect these byproducts reliably across various RNA sequences[5]. It serves as a robust indicator for the presence or absence of dsRNA contaminants. Additionally, techniques such as gel electrophoresis, high-performance liquid chromatography (HPLC), cryo-electron microscopy, and RNA sequencing are employed for qualitative dsRNA analysis. For quantitative assessment, methods like enzyme-linked immunosorbent assay (ELISA), homogeneous time-resolved fluorescence (HTRF) assay, MDA5 filament analysis, and ATPase activity measurement are used. A novel method, microfluidic electrophoresis, has recently been introduced for rapid, high-throughput analysis of dsRNA and mRNA fragments, enabling precise determination of their lengths through laddering10.

Both qualitative and quantitative analyses hinge on the accurate recognition of dsRNA byproducts by antibodies. However, differentiating true dsRNA byproducts from RNA's inherent secondary structures presents a significant challenge in antibody-based detection. To enhance detection sensitivity and accuracy, it is essential to utilize appropriate controls, such as synthetic RNA—with or without modifications—or other cost-effective alternatives like CATUG's dsRNA controls, ensuring the analysis accurately identifies dsRNA.

Table 1. CATUG dsRNA Products

Cat. NO.	Products Name
CT10000	CatPure™ dsRNA 700(UTP)
CT20000	CatPure™ dsRNA 700(N1mpU)
CT30000	CatPure™ dsRNA 1800(UTP)
CT40000	CatPure™ dsRNA 1800(N1mpU)
CT50000	CatPure™ dsRNA hairpin70(UTP)
CT60000	CatPure™ dsRNA hairpin70(N1mpU)

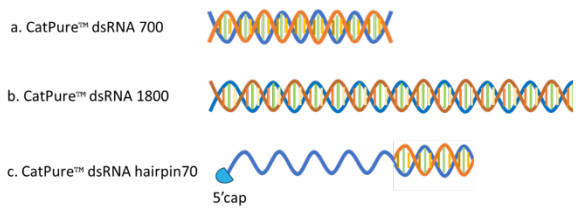

Figure 3. Schematic representation of the structure of CatPure™ dsRNA Controls.

CATUG CatPure™ dsRNA Products

CATUG offers a comprehensive selection of long dsRNA controls, developed as reliable standards for use in dsRNA detection assays. Currently, three variants are available for in vitro applications: dsRNA700, dsRNA1800, and dsRNA hairpin70, as summarized in Table 1. The dsRNA700 and dsRNA1800 products consist of extended double-stranded RNA molecules, while the dsRNA hairpin70

features a unique single-stranded RNA structure with a 3' terminal hairpin (Fig. 3).

Key Attributes of CatPure™ dsRNA Controls

(i) **Precision and Consistency**: CatPure™ dsRNA controls are designed to set industry standards by delivering high precision and consistency in every assay, ensuring dependable and reproducible results.

(ii) **Quality Assurance**: Manufactured under stringent quality standards, these controls support researchers in obtaining accurate and reliable data in dsRNA-focused studies. As demonstrated in Figures 4 and 5, the purity of CatPure™ dsRNA controls exceeded 80%, as validated by capillary electrophoresis (CE), agarose gel electrophoresis (AGE), and size-exclusion chromatography (SEC) analyses.

(iii) **Versatile Applications**: The broad applicability of CatPure™ dsRNA products makes them suitable for a wide range of research areas, underscoring their flexibility and reliability in diverse experimental settings.

(iv) **Advanced Detection Support**: These controls are fully compatible with leading dsRNA detection platforms, including ELISA, immunoblotting, and microfluidic electrophoresis, enabling robust and detailed dsRNA analysis across various conditions. For instance, as shown in Figure 6, CatPure™ dsRNA controls demonstrated significantly stronger binding affinity to the dsRNA-specific monoclonal antibody (J2) compared to Poly I: C. Moreover, no difference in binding affinity was observed among CatPure™ dsRNA controls of different molecular weights, indicating consistent antibody recognition across product variants.

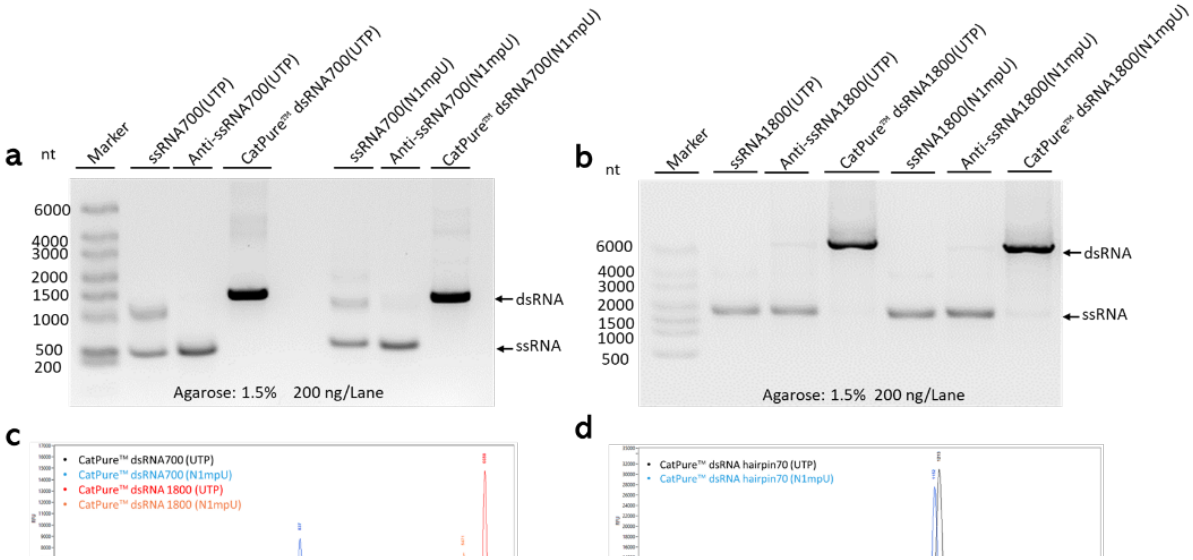

Figure 4. Agarose gel electrophoresis (AGE) and capillary electrophoresis (CE) of CatPure™ dsRNA Controls. ACE to detect CatPure™ dsRNA 700 (a) and CatPure™ dsRNA 1800 (b); CE to detect CatPure™ dsRNA 700 and CatPure™ dsRNA 1800 (c) and CatPure™ dsRNA hairpin70.

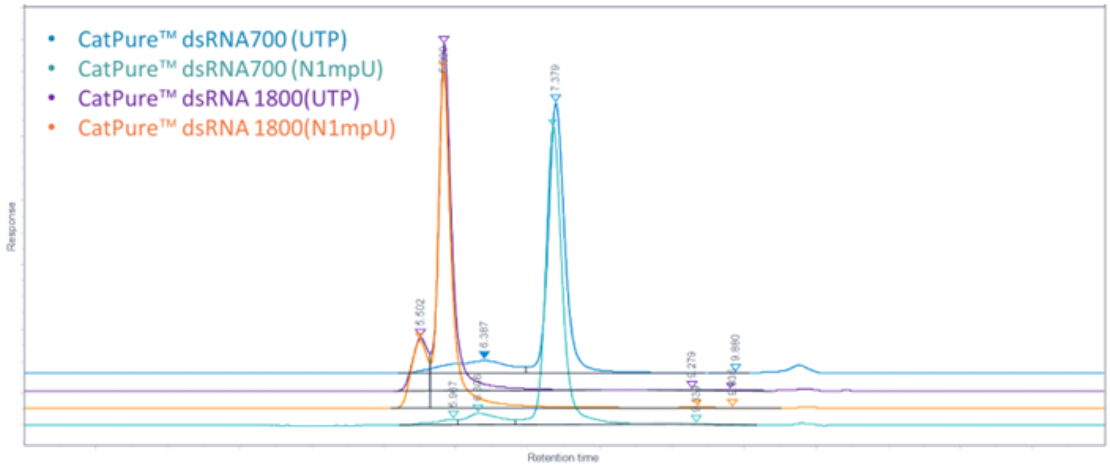

Figure 5. Size exclusion chromatography (SEC) of CatPure™ dsRNA Controls.

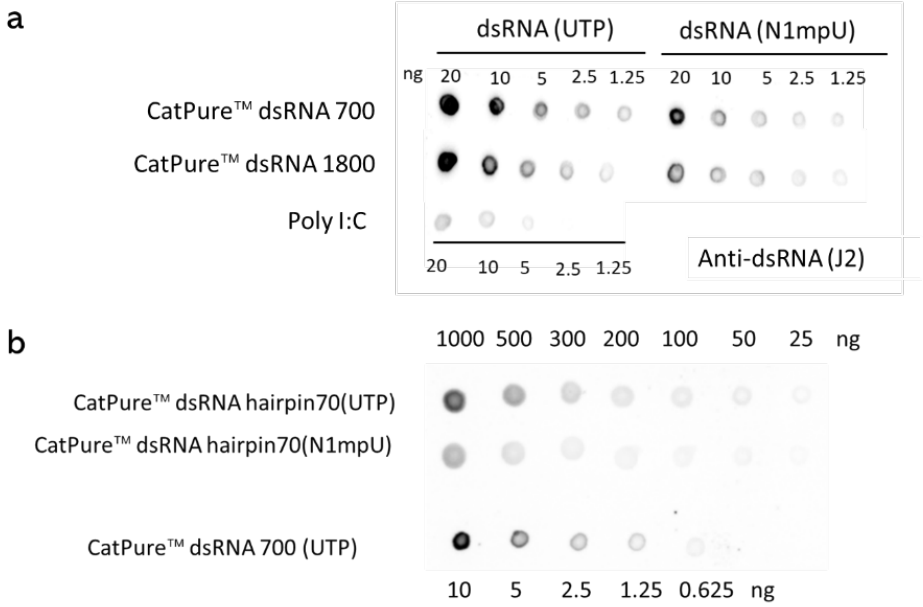

Figure 6. Representative Dot blot images to detect CatPure™ dsRNA Controls. CatPure™ dsRNA 700, CatPure™ dsRNA 1800 (a) and CatPure™ dsRNA hairpin 70 (b) detection by anti-dsRNA (J2) antibody.

Case Study 1: Comparison of Standard Curves Using Commercial and CatPure™ dsRNA Controls in ELISA-Based Quantification of dsRNA

ELISA is one of the most widely used methods for quantifying double-stranded

RNA (dsRNA) in mRNA products. A critical step in this process is the establishment of a standard curve using well-characterized dsRNA controls to ensure accurate and reliable quantification. In this study, both commercial dsRNA controls and CatPure™ dsRNA controls were used to generate standard curves for comparison.

Figure 7 illustrates notable differences between the resulting standard curves. Specifically, CatPure™ Std dsRNA 700(UTP) and CatPure™ Std dsRNA 700(N1mpU) showed responses comparable to those of the Commercial Std dsRNA 500(N1mpU), indicating similar performance in ELISA-based detection. In contrast, CatPure™ Std dsRNA 1800(UTP) and CatPure™ Std dsRNA 1800(N1mpU) exhibited lower responses relative to the Commercial Std dsRNA 500(N1mpU). These differences are further supported by the EC50 values summarized in Table 2: 0.473 for Commercial Std dsRNA 500(N1mpU), 0.470 for CatPure™ Std dsRNA 700(UTP), 0.767 for CatPure™ Std dsRNA 700(N1mpU), 1.806 for CatPure™ Std dsRNA 1800(UTP), and 1.785 for CatPure™ Std dsRNA 1800(N1mpU).

These results highlight that the accuracy of dsRNA quantification can be influenced by the length and chemical modifications of the dsRNA standards used, emphasizing the importance of selecting appropriate controls for precise analytical outcomes.

Table 2. EC50 of different dsRNA

Sample	EC_{50}(ng/mL)
Commercial dsRNA 500(N1mpU)	0.473
CatPure™ dsRNA 700(UTP)	0.470
CatPure™ dsRNA 700(N1mpU)	0.767
CatPure™ dsRNA 1800(UTP)	1.806
CatPure™ dsRNA 1800(N1mpU)	1.785

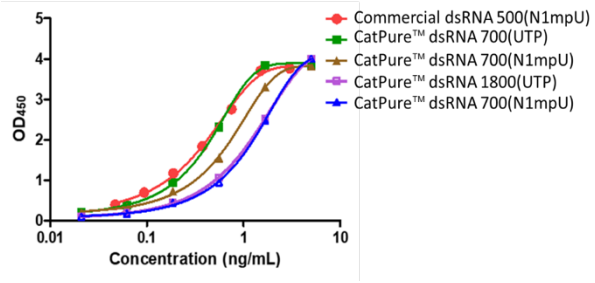

Figure 7. Comparison of different dsRNA controls by ELISA.

Case Study 2: Detection of dsRNA Using Enzyme-Based Enrichment Combined with Microfluidic Electrophoresis and CatPure™ dsRNA Controls

Researchers have developed a method that combines enzymatic enrichment with microfluidic electrophoresis to detect and characterize double-stranded RNA (dsRNA) impurities in mRNA samples. In this approach, the enzyme S1 nuclease is used to selectively degrade single-stranded RNA (ssRNA), while leaving dsRNA intact. The treated samples are then analyzed using microfluidic electrophoresis, which allows for the sensitive detection of low concentrations of dsRNA within complex mRNA samples.

The method was validated based on its ability to detect dsRNA of varying lengths and sensitivity levels. CatPure™ dsRNA controls of 700 bp and 1800 bp (1 ng/µL) were tested in combination with mRNA transcripts of different lengths—818 nt, 1198 nt, 1913 nt, and 4451 nt (200 ng/µL) (Figure 8). In all samples, mRNA was completely digested by S1 nuclease, while the dsRNA controls were preserved and detected with distinct migration profiles. This allowed for the simultaneous identification of multiple dsRNA species in a single sample without interference.

Further analysis of the 700 bp dsRNA control at concentrations of 1.0, 2.5, and 5.0 ng/µL demonstrated a strong correlation between peak area and dsRNA concentration, confirming the method's sensitivity. The lower detection limit was determined to be 0.32 ± 0.07 ng/µL.

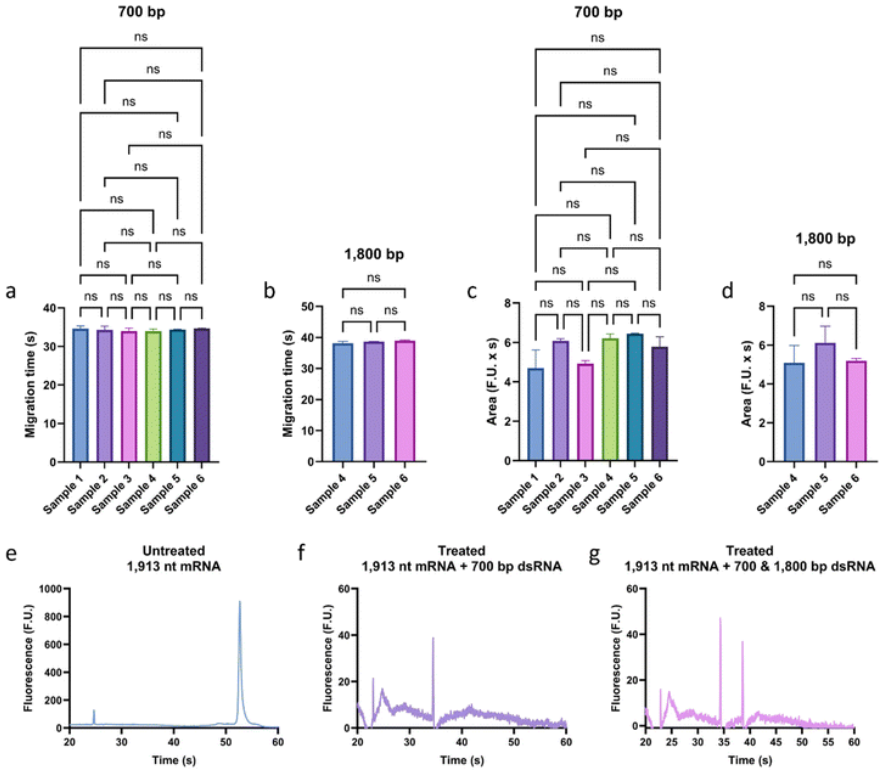

Figure 8. Assessing the robustness of the method against different mRNA lengths (200 ng/µL).

These results confirm that CatPure™ dsRNA controls are effective tools for supporting the development and validation of advanced dsRNA detection technologies.

Conclusion

Accurate detection of dsRNA byproducts is critical in the development and manufacturing of low-immunogenic mRNA therapeutics. CATUG is dedicated to advancing the quality of mRNA-based therapies by providing high-quality dsRNA standards, thereby supporting the process throughout mRNA drug development, manufacturing and quality control.

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