Application Note

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IVT mRNA Encapsulation Efficiency Assessment Using Agilent Fragment Analyzer Systems

Author

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Abstract

In vitro transcribed (IVT) mRNA has emerged in recent years as an innovative biotherapeutic platform. Development of these therapeutics involves synthesis of an IVT mRNA, referred to as the drug substance (DS), and subsequent formulation of the DS into a delivery vehicle, such as a lipid nanoparticle (LNP), creating the final drug product (DP). Given the complex and unique characteristics of these molecules, quality assessment at various stages of the workflow is essential for successful IVT mRNA biotherapeutic development.

Many critical quality attributes (CQAs) have been identified for evaluating the success of DP formulations, including integrity, purity, size, and encapsulation efficiency. Assessment of the encapsulation efficiency CQA is dependent upon reliable quantification of the IVT mRNA. Fluorescence-based plate readers are commonly employed for this assessment using RNA quantitation assays such as RiboGreen. This application note presents the Agilent Fragment Analyzer systems as an alternative method for encapsulation efficiency assessment. The systems use parallel capillary electrophoresis to separate the samples by size, and offer the resolution needed for integrity, purity, and sizing CQA analysis. The systems also allow for quantitative analysis and can be used to provide concentration measurements for both total and residual IVT mRNA in a DP[1]. The Fragment Analyzer enhances the IVT mRNA CQA workflow by consolidating necessary testing for characterizing IVT mRNA, including encapsulation efficiency, integrity, and sizing into a single instrument.

Introduction

Following the development of the first IVT mRNA vaccines for COVID-19, various regulatory agencies have issued recommendations and guidelines for the Critical Quality Attributes (CQAs) of IVT mRNA in vaccines and other biotherapeutic applications. These guidelines specifically address both the initial IVT mRNA, referred to as the drug substance (DS), and the subsequent LNP-encapsulated IVT mRNA, or the drug product (DP). The LNP protects the IVT mRNA from degradation in vivo, and enhances cellular uptake and translation efficiency.

One of the CQAs used to evaluate a successful DP formulation is calculation of the encapsulation efficiency. Reliable quantification of the IVT mRNA within the DP is necessary for this analysis, including both the amount of the DS that is encapsulated, as well as any residual IVT mRNA that fails to be encapsulated into the delivery vehicle.

To successfully measure the IVT mRNA that was encapsulated, the LNP must be disrupted to release the IVT mRNA prior to measurement[1], as demonstrated in Figure 1. The amount of encapsulated IVT mRNA can be calculated by subtracting the amount of residual IVT mRNA from the total IVT mRNA. Encapsulation efficiency is then determined by dividing the amount of encapsulated IVT mRNA by the amount of total IVT mRNA, as shown in Formula 1. Methods commonly used for sample quantification, such as the RiboGreen assay, are often used for the analysis of encapsulation efficiency. However, these methods can only quantify and do not distinguish between full length and degraded IVT mRNA, making it necessary to incorporate other analytical methods into the CQA workflow for full characterization of the DP.

The Fragment Analyzer provides both quantitative and qualitative analysis of nucleic acids through parallel capillary electrophoresis. Among the many CQAs tested, the Fragment Analyzers are used to analyze the size, integrity, and purity of IVT mRNA DSs and DPs[1,2,3,4]. The system also offers the ability to calculate the total concentration of a sample. Quantification of the residual and total IVT mRNA enable the determination of encapsulation efficiency. The system enhances the IVT mRNA CQA workflow by consolidating the amount of testing necessary to characterize both the IVT mRNA DS and DP. This application note provides an alternate method for determining encapsulation efficiency, while also providing information for other CQAs of IVT mRNA biotherapeutics.

$$\%\ Encapsulation\ Efficiency = \frac{(Concentration_{\ Total\ IVT\ mRNA} - Concentration_{\ Residual\ IVT\ mRNA})}{Concentration_{\ Total\ IVT\ mRNA}} \times 100$$

Formula 1. Encapsulation efficiency percentage.

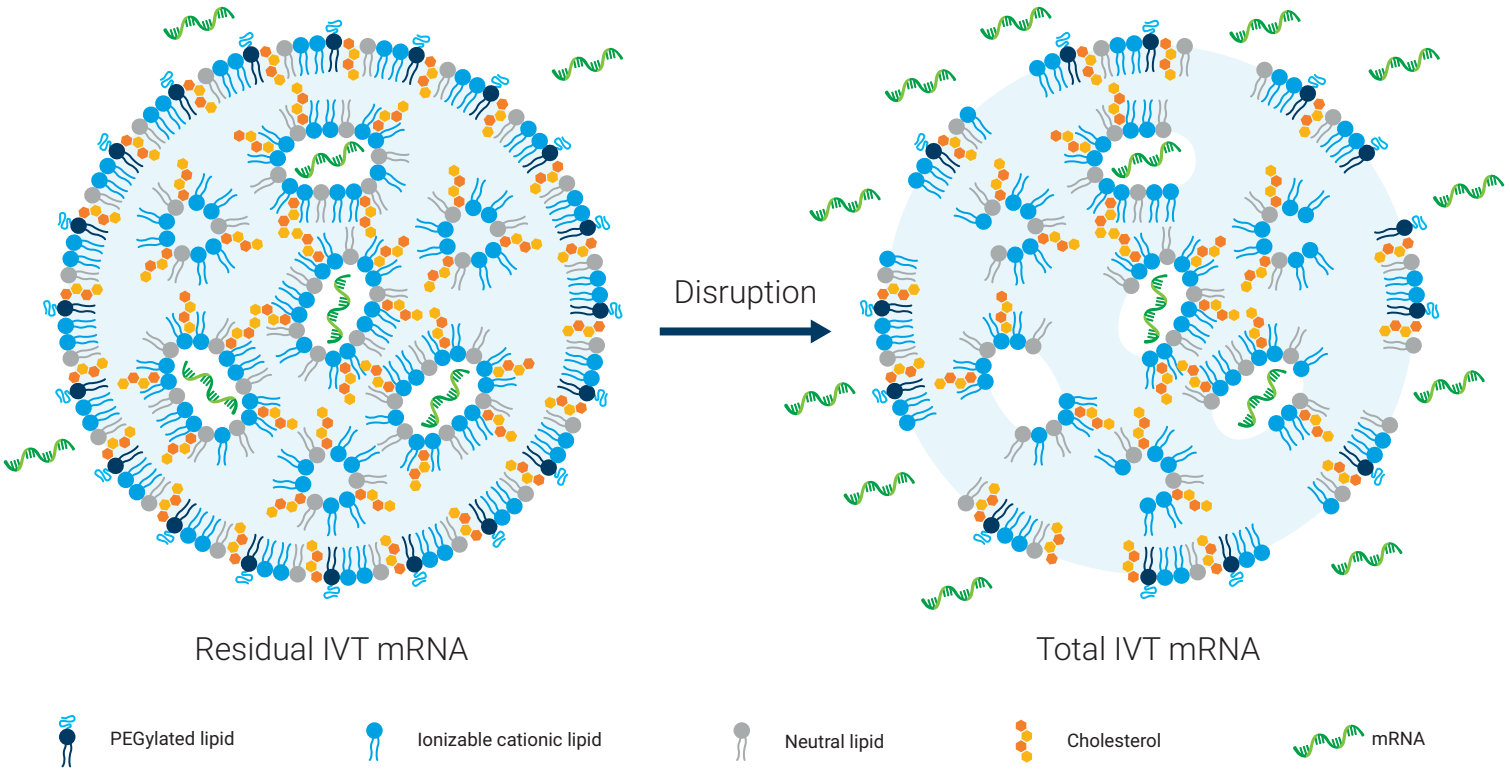


Figure 1. Graphical representation of an LNP-encapsulated IVT mRNA, or drug product, with residual IVT mRNA outside of the LNP. Following disruption of the LNP, the IVT mRNA is released for assessment of the total IVT mRNA used in the formulation process.

Methods

IVT mRNA precursor and LNP-encapsulated IVT mRNA encoding for Firefly Luciferase (Fluc) (mRNA-Cap1+LNP [Firefly Luciferase], PackGene Biotech, Inc.) samples were used in this study to mimic an IVT mRNA DS and DP, respectively. The DP was disrupted with surfactant for 20 minutes at room temperature at a concentration of 10 ng/µL to release the IVT mRNA from the LNP. All samples were further diluted to a range of 10 to 2.5 ng/µL with nuclease-free water. Aliquots of the DP before and after LNP disruption were assessed on an Agilent 5300 Fragment Analyzer system with the Agilent HS RNA kit (DNF-472) using the IVT mRNA method B[4,5]. The Agilent ProSize data analysis software was used to assess the total concentration of each of the samples, which is automatically calculated and reported in the peak table. The concentration of the DP before disruption of the LNP is representative of the amount of unencapsulated or residual IVT mRNA, while after disruption is the concentration of the total IVT mRNA within the DP. Simultaneously, the samples were analyzed with the RiboGreen assay (Quant-it RiboGreen RNA Assay Kit, Thermo Fisher Scientific, p/n R11490) using the Agilent BioTek Synergy LX multimode reader equipped with a Green Filter Cube (p/n 1505005). Encapsulation efficiency with both systems was calculated using Formula 1. The composition of other IVT mRNA samples and delivery systems may differ and are often proprietary, and therefore may require optimization of the disruption and analysis methods.

Results and discussion

IVT mRNA drug product assessment

The RNA kits for the Fragment Analyzer have been optimized for assessment of IVT mRNA[4,5], including DS and DP size, purity, and integrity. Encapsulation of the DS into a delivery vehicle such as an LNP is a crucial step in IVT mRNA biotherapeutics, allowing for protection and delivery of the IVT mRNA in vivo. However, assessment of the DP is complicated because the LNP prevents access to the IVT mRNA for evaluation. To accurately assess the encapsulated IVT mRNA with the Fragment Analyzer, the LNP must be disrupted to release the IVT mRNA, as shown in Figure 1. Analysis of the sample before disruption results in a small peak at the expected size, indicating the presence of free residual IVT mRNA that was not encapsulated in the LNP. After disruption of the LNP and release of the IVT mRNA, the peak height of the IVT mRNA increases substantially (Figure 2).

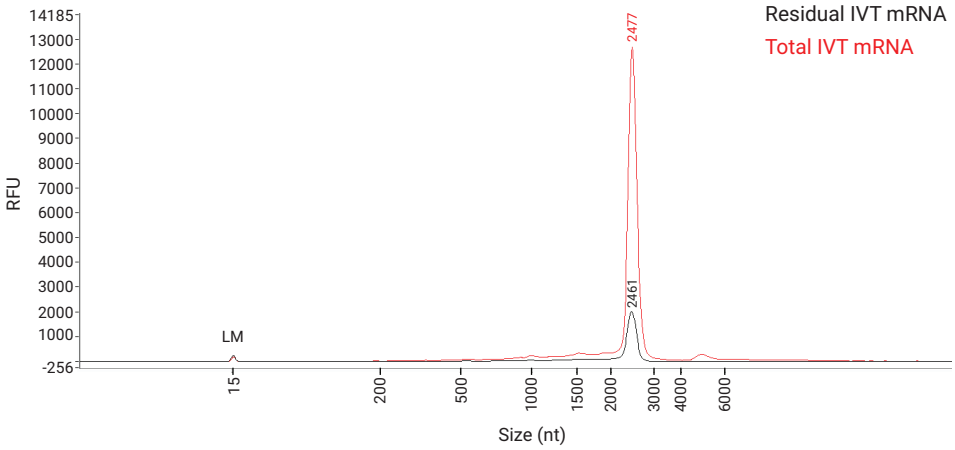


Figure 2. Analysis of an LNP-encapsulated IVT drug product using the Agilent Fragment Analyzer system, before (black) and after (red) disruption of the LNP.

Accurate quantification of IVT mRNA with fluorescent methods can be complicated, but the Fragment Analyzer provides precise and reliable relative quantification results[2]. To demonstrate this, a Fluc IVT mRNA DP was analyzed in multiple wells and across three subsequent runs before and after disruption of the LNP. Figure 3A highlights the precision of the quantification values, with less than 11% CV across all three runs. Assessment of multiple replicates of the released IVT mRNA within a single run displayed very good precision at 3.3% CV (N=11) indicating reproducible results between capillaries.

The Fragment Analyzer also provides excellent linearity of quantification, as demonstrated in Figure 3B. Dilutions of the Fluc DP before and after LNP disruption were assessed on the Fragment Analyzer system using the HS RNA IVT mRNA method B. Both samples showed high R^2 values of 0.96 for the residual IVT mRNA and 0.99 for the disrupted DP. Together, this data demonstrates that the Fragment Analyzer provides robust and reliable quantification analysis that is necessary for the calculation of encapsulation efficiency.

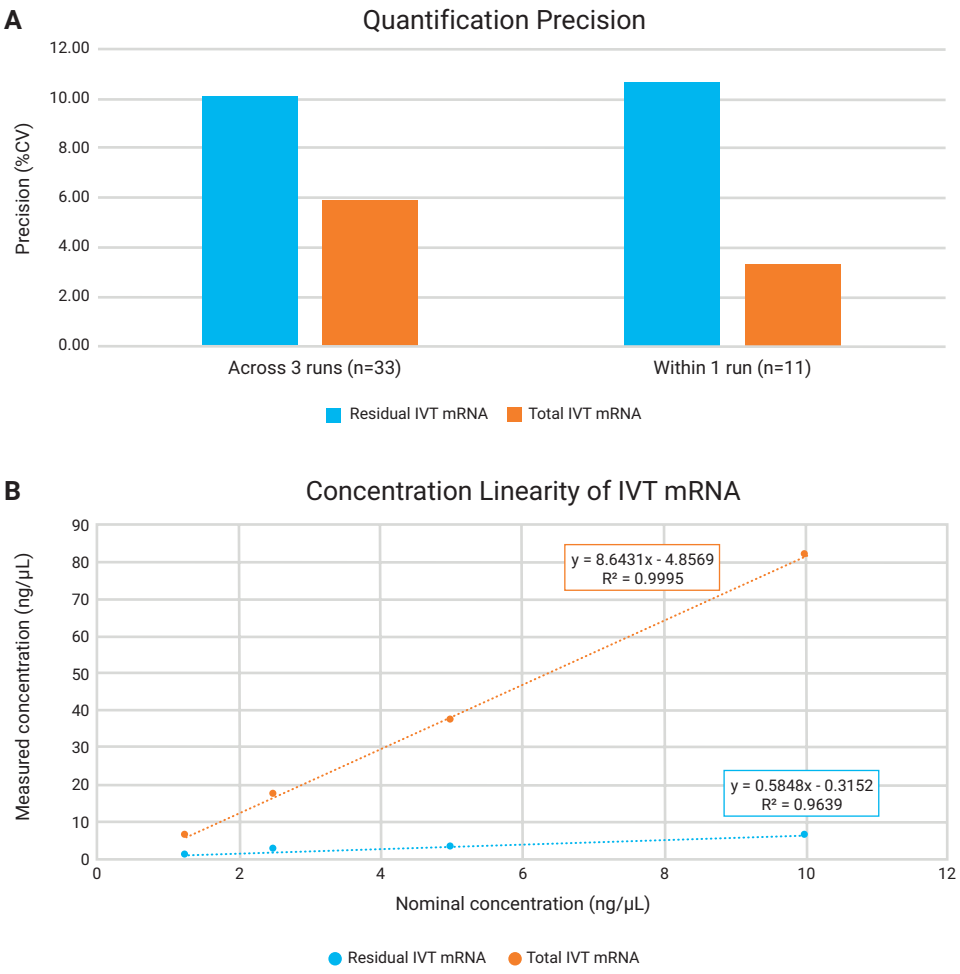
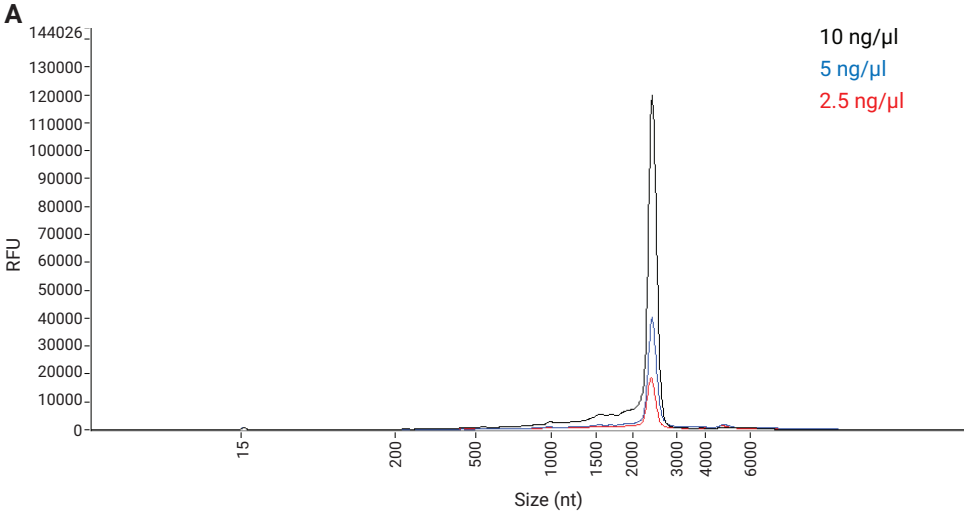


Figure 3. A) Precision and B) linearity of quantification using the Agilent Fragment Analyzer systems and Agilent HS RNA kit with the IVT mRNA method B. Total IVT mRNA refers to the residual IVT mRNA and the encapsulated IVT mRNA, following disruption of the LNP.

Encapsulation efficiency

The concentration of the residual and total IVT mRNA determined by the Fragment Analyzer systems was used to calculate the percent encapsulation efficiency. For comparison, the same samples were also assessed with the RiboGreen assay. A serial dilution of the samples from 10 to 2.5 ng/µL showed comparable results between the two assays (Figure 4A). The RiboGreen assay displayed an encapsulation efficiency of 94 to 97% across the concentration range. The data from the Fragment Analyzer was consistent at 10 and 5 ng/µl, with an encapsulation efficiency of 92% (Figure 4B) and decreased slightly to 86% at the lower range of the kit specifications. The loading concentration for IVT mRNA is important to appropriately visualize the impurities within a sample[2,3]. As seen in the electropherogram overlays of the sample at different concentrations, more impurities are visualized when the sample is loaded at a higher concentration. Lower concentrations may not detect as many impurities, thereby affecting the sample concentration and encapsulation calculations. In contrast, the Ribogreen assay can not distinguish between IVT mRNA and impurities, and the reported concentration is therefore the total of all RNA in the sample, including any impurities. For best practices with the Fragment Analyzer, it is recommended to load IVT mRNA samples within the mid- to high range of the kit specifications[2,3].

A



B

	Encapsulation Efficiency %	
Nominal concentration	**RiboGreen**	**Fragment Analyzer**
10 ng/µl	94.2	92.4
5 ng/µl	97.8	92.0
2.5 ng/µl	97.5	86.0

Figure 4. The Agilent Fragment Analyzer system was used to assess a serial dilution of the Fluc IVT mRNA drug product following disruption of the LNP. A) Electropherogram overlay of the samples analyzed with the Agilent HS RNA kit using the IVT mRNA method B. B) The encapsulation efficiency across the concentration range of the Fragment Analyzer was compared to the RiboGreen assay. N=2 per concentration.

The Fluc DP was further assessed on the HS RNA kit using the IVT mRNA method B at 5 ng/µl in multiple replicates. The encapsulation efficiency was calculated for each replicate, with an average of 90% efficiency (Figure 5). The measurements showed excellent precision at 1.2% CV. Further, the same sample plate was injected multiple times to demonstrate the robustness and reproducibility of the system and showed similar percentages across three subsequent analyses. In all cases, the encapsulation efficiency was consistently at 90%, comparable to the average RiboGreen measurement of 91% (Figure 5).

Additionally, the encapsulation efficiency was calculated for multiple samples prepared in different days. As shown in Figure 6, the encapsulation efficiency of the Fluc DP at 5 ng/µl was calculated with both the RiboGreen assay and the Fragment Analyzer on two separate days. Notably, the encapsulation efficiency between days with the RiboGreen assay was much higher than that of the Fragment Analyzer. The RiboGreen assay reported 98% on day 1 and decreased to 91% on day 2. In contrast, the Fragment Analyzer displayed an encapsulation efficiency of 92% on day 1 and 90% on day 2. These comparisons demonstrate that the Fragment Analyzer is an acceptable and reliable method for calculating encapsulation efficiency within the IVT mRNA drug product CQA workflow.

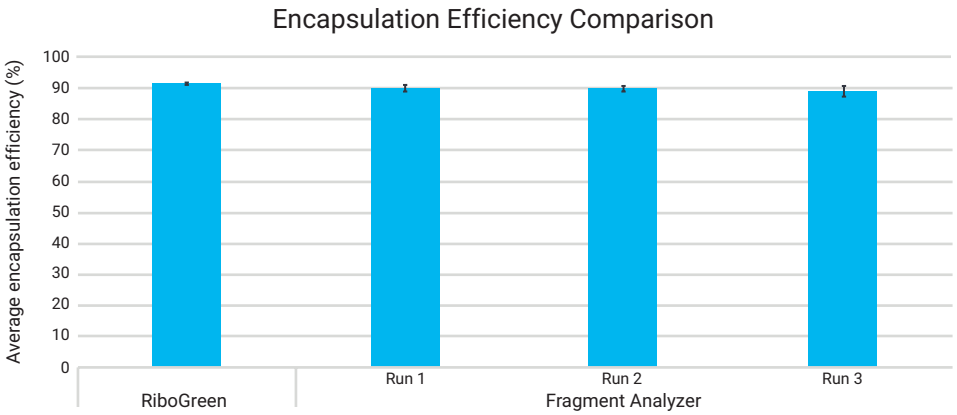


Figure 5. The encapsulation efficiency of the Flu IVT mRNA drug product was assessed across multiple runs of the same plate on the Agilent Fragment Analyzer system and compared to the RiboGreen assay. The sample was prepared at 5 ng/µL. Fragment Analyzer N=11 wells per run. RiboGreen, N=7 replicates. Error bars represent standard deviation.

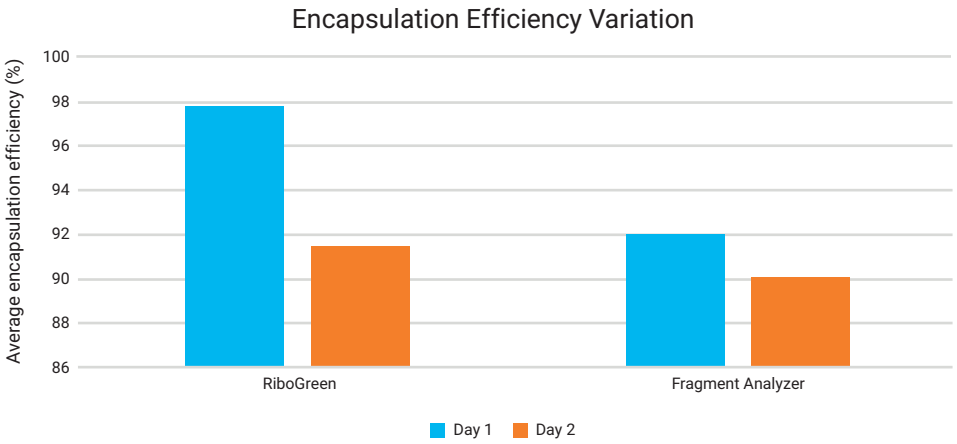


Figure 6. The encapsulation efficiency of the Fluc IVT mRNA drug product was assessed across multiple preparations on the Agilent Fragment Analyzer system and with the RiboGreen assay. Day 1: RiboGreen and Fragment Analyzer N=2, Day 2: RiboGreen N=7, Fragment Analyzer N=11.

Conclusion

The Agilent Fragment Analyzer systems perform both quantitative and qualitative nucleic acid analysis using parallel capillary electrophoresis and are routinely used in the characterization of IVT mRNA DS and DP. CQAs commonly assessed with the systems include integrity, size, and purity. As demonstrated with the Fluc example in this application note, the systems can also be used to quantify residual and total IVT mRNA for encapsulation efficiency, providing an alternative use for the Fragment Analyzer systems. The systems can be used to streamline the IVT mRNA CQA workflow, consolidating essential tests for characterizing IVT mRNA onto one platform.

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www.agilent.com/genomics/fragment-analyzer

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