Application Note

Genomics

Agilent

Trusted Answers

Suitability Testing for IVT mRNA Analysis Using Agilent Fragment Analyzer Systems

Authors

Tim Butler, Sheng-Yuan Huang, Kyle Luttgeharm, Marko Marinkovich, and Whitney Pike,
Agilent Technologies, Inc.

Abstract

The development of drugs and vaccines necessitates rigorous analytical procedures to ensure final product quality. Variations in instrument settings, sample characteristics, users, and method parameters can significantly impact analytical results. Evaluating these variables is crucial for understanding and minimizing sources of variability, thereby validating and implementing analytical methods throughout the product life cycle. This application note provides an example of a suitability study using the Agilent Fragment Analyzer system, an automated capillary electrophoresis system used to assess the size, concentration, and integrity of in vitro transcribed (IVT) mRNA. The study confirmed excellent system, method, and intermediate precision for integrity analysis. Additionally, the Fragment Analyzer showed strong linearity across a broad concentration range and demonstrated its stability-indicating capability. These findings highlight the robustness and precision of the Fragment Analyzer system, demonstrating its suitability for use in the biopharmaceutical life cycle.

Introduction

The biopharmaceutical life cycle involves the transition of a drug substance from the initial research phases through clinical production. As such, development of drugs and vaccines requires many analytical procedures and methods to ensure final product quality. To guide this process, regulatory agencies such as the United States Pharmacopeia (USP) have established guidelines for the assessment of various critical quality attributes (CQAs) for drug substances and drug products.[1] These guidelines include possible methods to test specific attributes. For example, in in vitro transcribed (IVT) mRNA vaccines, the integrity of the drug substance and drug product can be assessed using either agarose gel electrophoresis (AGE) or capillary electrophoresis (CE).

Many procedures proposed for CQA assessment are inherently influenced by factors that can affect analytical results, such as variations in instrument settings, sample characteristics, method parameters, individual users, and laboratories. Evaluating how these variables can impact method performance helps scientists understand and reduce sources of variability within analytical procedures, allowing them to be validated and implemented into the product life cycle.

Life cycle management of analytical methods begins in R&D, where robust methods are developed. Subsequently, these methods are routinely assessed in quality control laboratories for trend analysis, enabling users to identify sources of variability that could indicate issues with the sample preparation or method. This application note illustrates the suitability studies that can be performed throughout a drug's life cycle, using the Agilent Fragment Analyzer system as an example. The Fragment Analyzer is an automated CE system used for assessing the integrity of IVT mRNA.[2] By testing potential variations in the procedure, this study demonstrates the system's suitability as an analytical method throughout the biopharmaceutical life cycle.

Experimental

A commercially available IVT mRNA encoding for Firefly Luciferase (Fluc), CleanCap FLuc mRNA (5moU) (TriLink Biotechnologies, p/n L-7202) was obtained and diluted to the appropriate concentration using nuclease-free water. Single-use aliquots were prepared and stored at –80 °C. Aliquots of the sample were prepared for analysis according to the Agilent HS RNA kit (DNF-472) manual, unless otherwise described. Samples were assessed on Agilent 5200 and 5300 Fragment Analyzer systems with the Agilent HS RNA kit using IVT mRNA method A.[3] Agilent ProSize data analysis software was used to assess the percent total of the main peak in the sample using the smear analysis function.

Results

System precision

The precision of the Fragment Analyzer system was demonstrated by assessing the integrity of a Fluc IVT mRNA across multiple capillaries within a single run. The sample was diluted to 2 ng/µL, prepared as a master mix with the HS RNA diluent marker, and aliquoted to six wells of a microplate. The plate was then analyzed with the HS RNA method A for IVT mRNA. The smear analysis function in the ProSize data analysis software was used to calculate the percent total of the main peak as an indicator of the integrity of the sample. The precision of the system was determined by calculating the coefficient of variation (%CV) across the run. This process was repeated three times, for a total of 18 data points. On average, the Fluc IVT mRNA showed a percent total of 81%. Each master mix displayed excellent precision within the run, with less than 0.4 %CV (Table 1). This finding is further demonstrated by an electropherogram overlay, showing the overlapping profiles of the six wells within a single run (Figure 1).

Table 1. Precision of the Agilent Fragment Analyzer system demonstrated by the average percent total of a Fluc IVT mRNA. N = 18 (three master mixes [MM] of sample and diluent marker were prepared, and six measurements taken from each MM).

	%Total			
	MM 1	MM 2	MM 3	
Well 1	80.6	81.1	81.3	
Well 2	81.0	80.6	81.7	
Well 3	81.4	81.5	81.6	
Well 4	80.9	81.2	81.3	
Well 5	81.1	81.3	81.5	Overall Average
Well 6	80.9	80.9	81.9	
Average	**80.98**	**81.10**	**81.55**	**81.21**
StDev	**0.26**	**0.32**	**0.23**	**0.36**
%CV	**0.33**	**0.39**	**0.29**	**0.44**

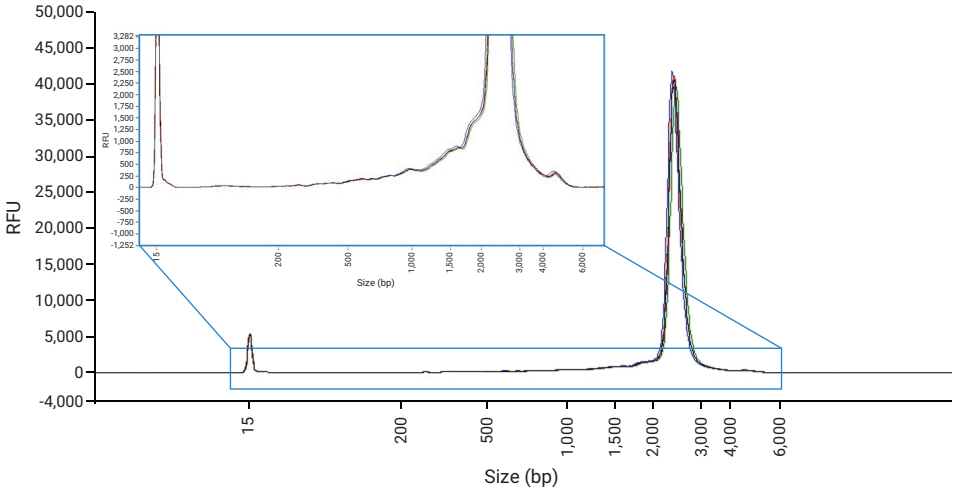

Figure 1. Fluc IVT mRNA analyzed on the Agilent Fragment Analyzer system, with multiple replicates overlaid to demonstrate system precision (N = 6).

Method precision

The reproducibility of the HS IVT mRNA method for the Fragment Analyzer was demonstrated by assessing the integrity of multiple replicates of a Fluc IVT mRNA. The sample was diluted to 2 ng/µL, and six individual aliquots of the sample were each individually mixed with HS RNA Diluent Marker in the sample plate. The plate was injected once on the Fragment Analyzer and the integrity of the sample calculated using the smear analysis function (Table 2). The sample had an average percent total of 82.30%. An overlay of the six individually prepared samples is shown in Figure 2. As shown in the inset image, the profiles are highly similar with minor fluctuations in signal intensity due to independent dilutions. Still, the samples displayed an excellent precision of 0.37 %CV, illustrating the reliability of the HS IVT mRNA method.

Table 2. Precision of the HS RNA kit method for the Agilent Fragment Analyzer system demonstrated by the average percent total of a Fluc IVT mRNA. N = 6 independent measurements.

	%Total
Well 1	82.5
Well 2	82.2
Well 3	81.8
Well 4	82.3
Well 5	82.3
Well 6	82.7
Average	**82.30**
StDev	**0.30**
%CV	**0.37**

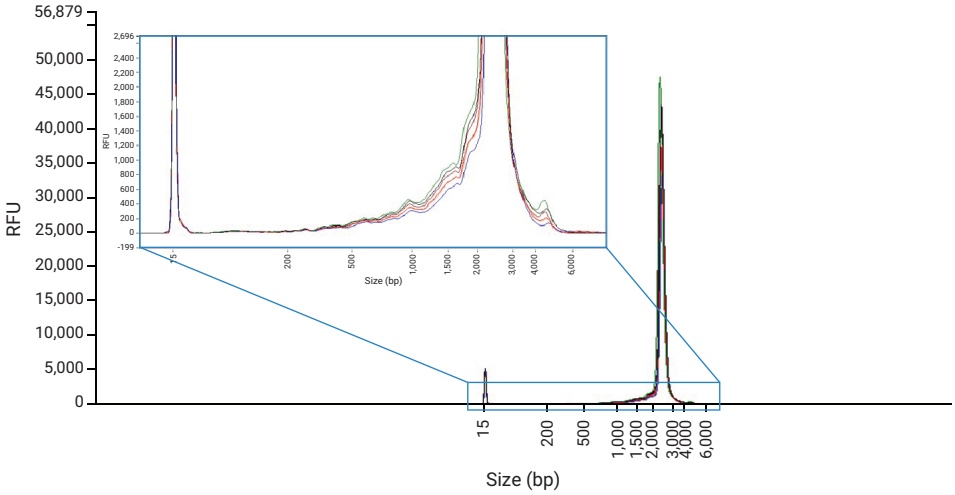

Figure 2. Fluc IVT mRNA analyzed on the Agilent Fragment Analyzer system, with multiple replicates overlaid to demonstrate method precision (N = 6).

Ruggedness (intermediate precision)

Transitioning RNA drug substances from initial research phases to clinical production requires testing analytical methods across diverse variables. In this study, the IVT mRNA method for the Fragment Analyzer was evaluated by five different users, with multiple systems across two different laboratory settings, for a total of 14 instances, as summarized in Table 3.

Table 3. Results of intermediate precision testing for the Agilent Fragment Analyzer system. Each instance details the specific user, lab location, and instrument that was used for analysis. The data was assessed using two smear analysis regions, one with the individual user setting the smear boundaries to encompass the main peak of the sample and the second with a preset range of 2,000 to 3,000 nt.

Instance	User #	Lab #	Instrument #	% Total with Smear Set Individually	% Total with 2,000 – 3,000 nt Smear
1	1	1	1	84.70	84.25
2	1	2	2	85.00	85.05
3	2	1	1	84.03	82.33
4	2	1	3	83.20	82.52
5	2	2	2	86.63	84.53
6	3	1	3	84.92	88.12
8	3	2	2	82.40	83.98
9	4	1	1	80.00	80.65
10	4	1	3	82.23	85.70
11	4	2	2	80.90	82.47
12	5	1	1	80.98	82.73
13	5	1	1	81.10	83.15
14	5	1	1	81.55	83.15
			Average	**82.90**	**83.74**
			%CV	**2.44**	**2.24**

IVT mRNA integrity was determined using a smear analysis. The ProSize data analysis software used by the Fragment Analyzer allows users to set specific size ranges and automatically calculates the percent total of the sample within that range, providing an indication of sample integrity. However, determining where to place smear boundaries can be difficult, and many users apply their own criteria for establishing where to set them. In this study, each of the five analysts set the smear boundaries to encompass the main fragment within the sample. Additionally, a second smear analysis was set at 2,000 to 3,000 nt to provide a standard analysis and exclude any user variability in terms of data analysis. Across all 14 instances, the sample's integrity was measured at approximately 83% with both the standard and individually set smear analyses. The measurements were highly precise, with less than 2.4 %CV.

In-depth examination of the analyses by the different variables can provide users with insight into any potential issues and instill confidence in the system and method. For example, analysis of this dataset by instrument, using the individually set smear ranges, shows average integrities of 82.71 (instrument 1), 84.01 (instrument 2), and 85.45 (instrument 3) percent total for each of the three instruments, with a precision of 2.28, 3.07, and 1.63 %CV, respectively (Figure 3A). Analysis of the data set by user shows an average of 81.21 to 84.85 percent total (Figure 3B), with precision ranging from as low as 0.25 up to 2.13 %CV. This variability likely reflects differences in user set up, such as sample preparation or placement of the smear boundaries. While the analysis of IVT mRNA can be challenging, the Fragment Analyzer offers flexible analysis and provides highly reliable data across multiple users, instruments, and laboratories.

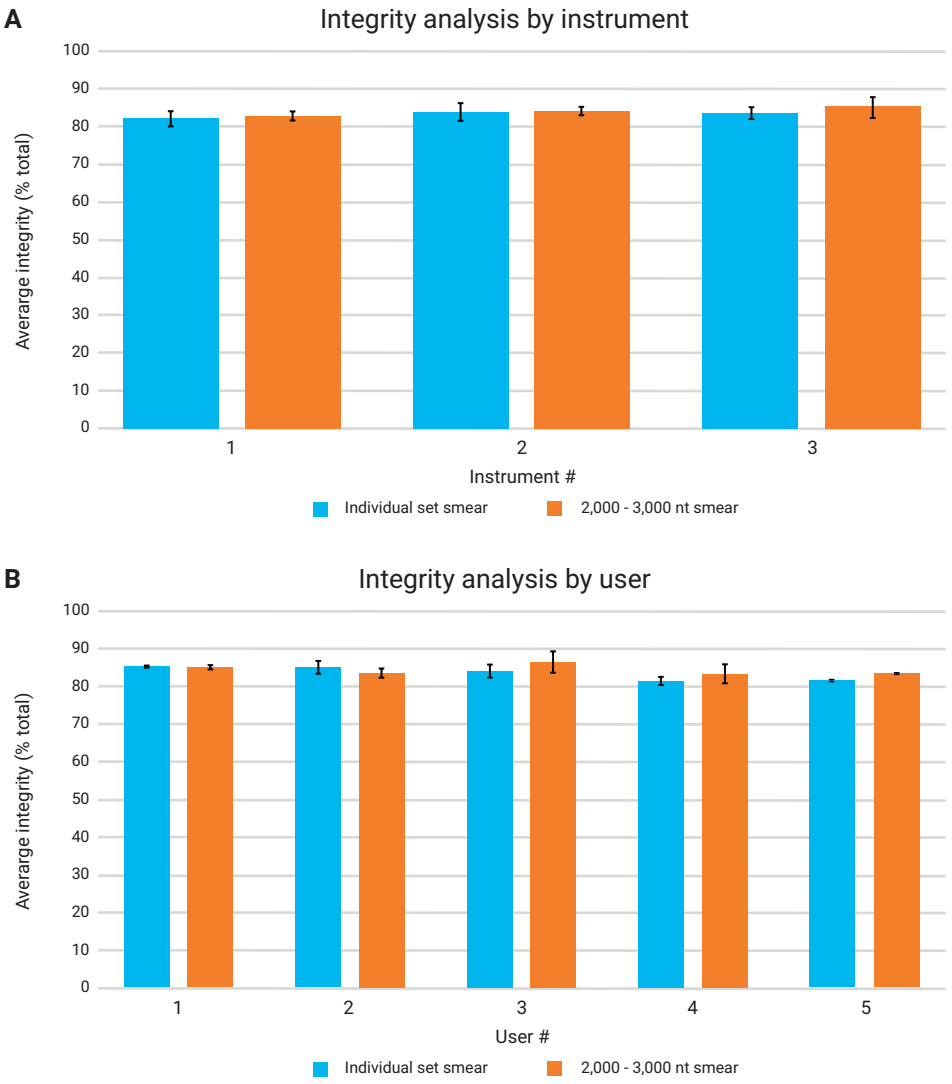

Figure 3. Results of intermediate precision testing for the Agilent Fragment Analyzer system. Testing was performed by five individual users, in two separate laboratories, using three different instruments. The data were assessed using two smear analysis regions, one with the individual user setting the smear boundaries to encompass the main peak of the sample, and the second with a preset range of 2,000 to 3,000 nt. For comparison, the tables demonstrate the average performance by (A) instrument and (B) user. Error bars represent standard deviation.

Degradation series

To demonstrate that the Fragment Analyzer is stability-indicating, a degradation series was prepared and analyzed using the HS RNA kit. Figure 4 shows an electropherogram overlay of the sample before and after heating at 90 °C for 1 to 9 minutes. The integrity of the intact sample was determined using a smear analysis encompassing the main peak. The same smear analysis settings used for the intact sample were used for each of the samples in the series, since clear boundaries could not be identified following intentional degradation. The intact sample showed an average integrity of 82.3%, which decreased with each minute of heat applied, demonstrating that the Fragment Analyzer is stability-indicating.

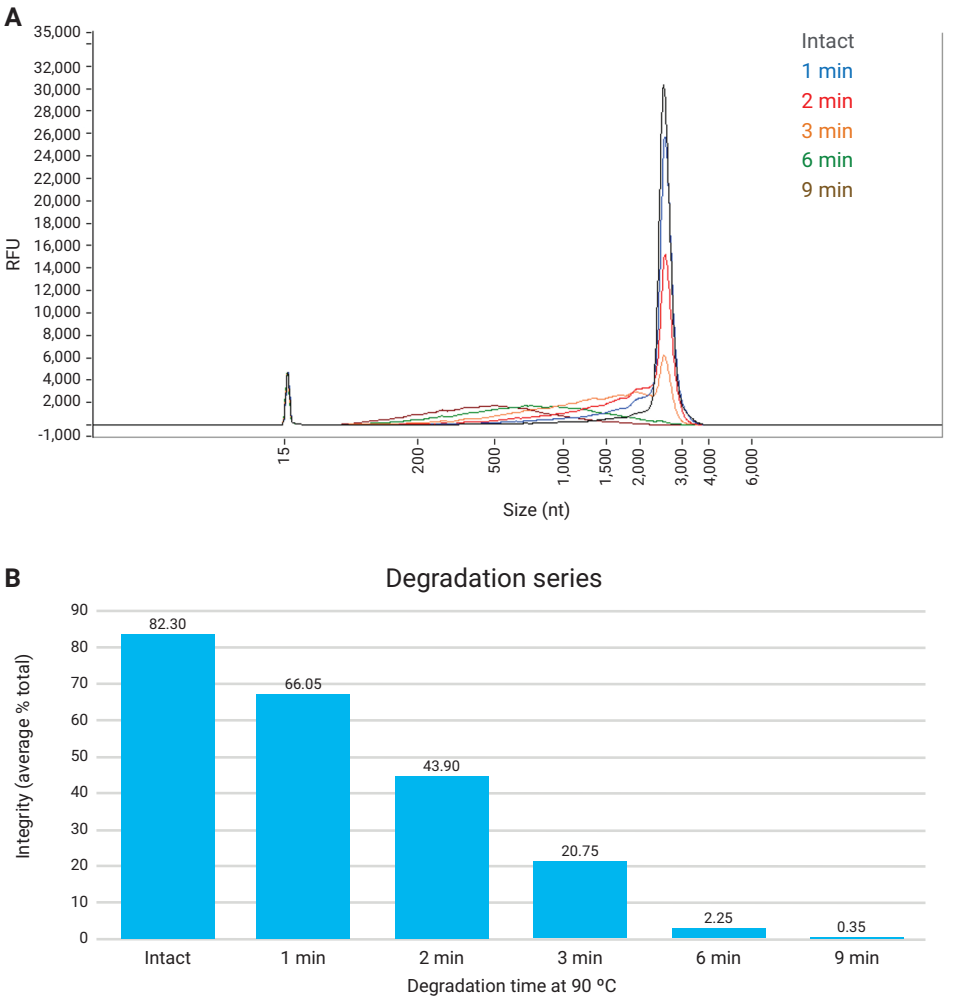

Figure 4. Degradation series of Fluc IVT mRNA analyzed on the Agilent Fragment Analyzer system with the Agilent HS RNA kit. (A) Electropherogram overlay of the intact RNA and following heat treatment at 90 °C for 1 to 9 minutes. (B) Average integrity of the samples throughout the degradation series. N = 2 replicates per time point.

Linearity

To determine the ability of the Fragment Analyzer to provide results proportional to sample concentration, dilutions of the Fluc mRNA were assessed for both integrity and concentration. An electropherogram overlay of the sample prepared using the HS RNA kit at concentrations ranging from 25 to 175% of the initial loading concentration (1.5 ng/µL) is shown in Figure 5A. The average percent total for each dilution was consistent, ranging from 84.9% to 86.4% (Figure 5B). The measurements showed excellent precision between replicates, with less than 0.8 %CV for each concentration. The Fragment Analyzer also showed strong linearity across the sample dilution concentrations, as demonstrated by an R^2 of 0.9928 (Figure 5C). These results confirm that the Fragment Analyzer, when used with the HS RNA kit and IVT mRNA method A, is suitable for analysis over a large concentration range and is linear from 25 to 175% of the nominal load. Similar results were achieved with the IVT mRNA method for the RNA kit (DNF-471), indicating that both methods can be used successfully for IVT mRNA integrity analysis (data not shown).

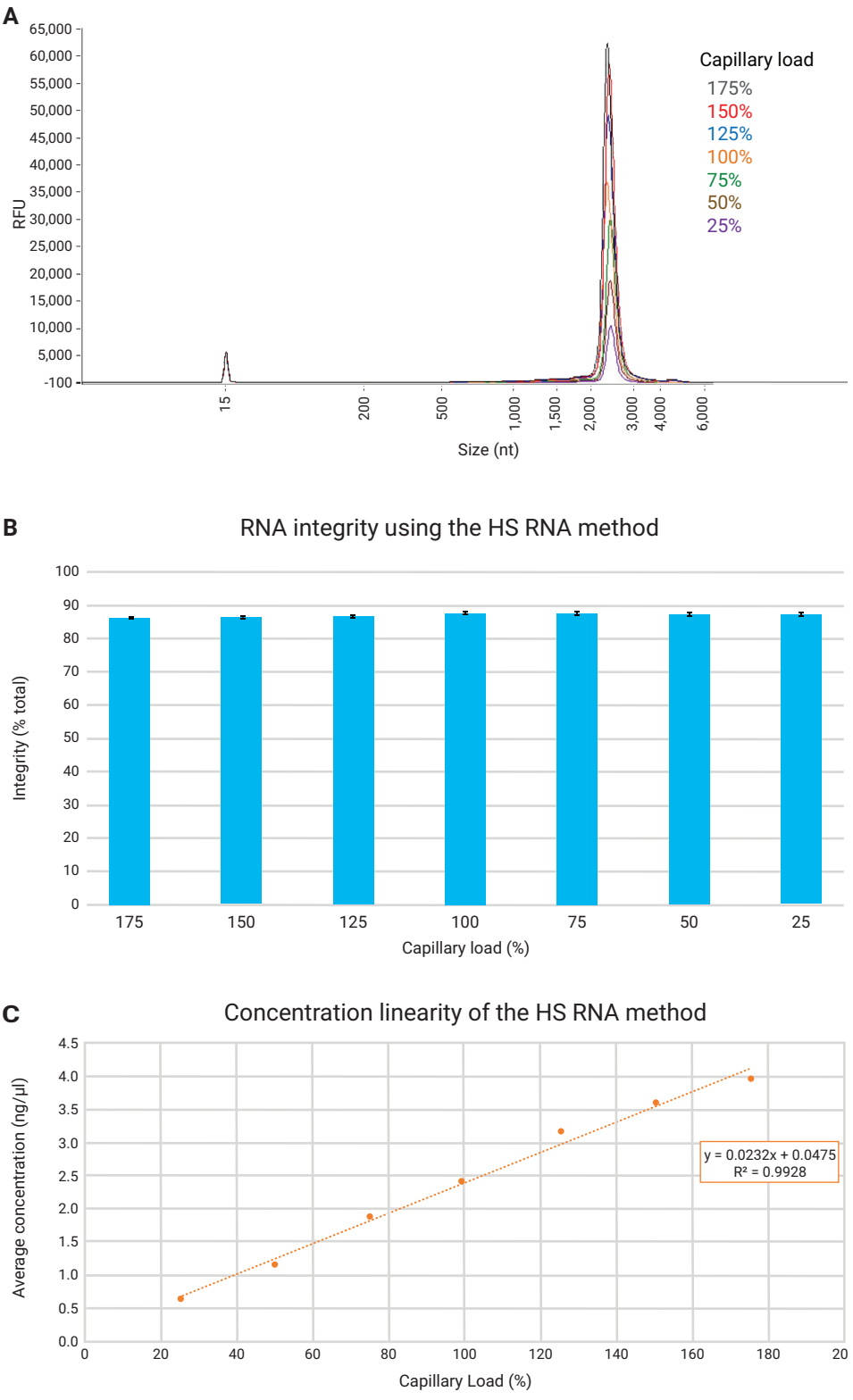

Figure 5. Fluc IVT mRNA analyzed on the Agilent Fragment Analyzer system with the Agilent HS RNA kit and IVT mRNA method A, across concentrations ranging from 25 to 175% of the initial loading concentration (1.5 ng/µL). (A) Electropherogram overlay of each concentration. (B) Integrity analysis of the sample across the concentration range. (C) Concentration linearity across the capillary loading range.

Conclusion

The Agilent Fragment Analyzer system, using the Agilent HS RNA kit, demonstrated robust performance in assessing the integrity of IVT mRNA across various analytical conditions. Both system and method precision were confirmed with excellent %CV values. Intermediate precision across different users, systems, and laboratory settings revealed consistent results. Linearity tests indicated strong proportionality of results to sample concentration. Additionally, the stability-indicating capability of the system was validated through a degradation series. These findings highlight the suitability of the Fragment Analyzer for reliable and precise analysis of IVT mRNA, providing an example of suitability testing performed throughout the biopharmaceutical life cycle.

References

1. Analytical Procedures for mRNA Vaccine Quality Draft Guidelines: 3rd edition. www.usp.org.The United States Pharmacopeial Convention, **2024**.
2. High-Sensitivity IVT mRNA Analysis Using the Agilent Fragment Analyzer Systems. *Agilent Technologies technical overview*, publication number 5994-7644EN, **2024**.
3. Agilent DNF-472 (15 nt) HS RNA Kit – IVT mRNA. *Agilent Technologies quick guide*, publication number D0117260, **2025**.

www.agilent.com/genomics/fragment-analyzer

For Research Use Only. Not for use in diagnostic procedures.

PR7001-4743

This information is subject to change without notice.

Published in the USA, July 2, 2025
5994-8480EN

Agilent
Trusted Answers