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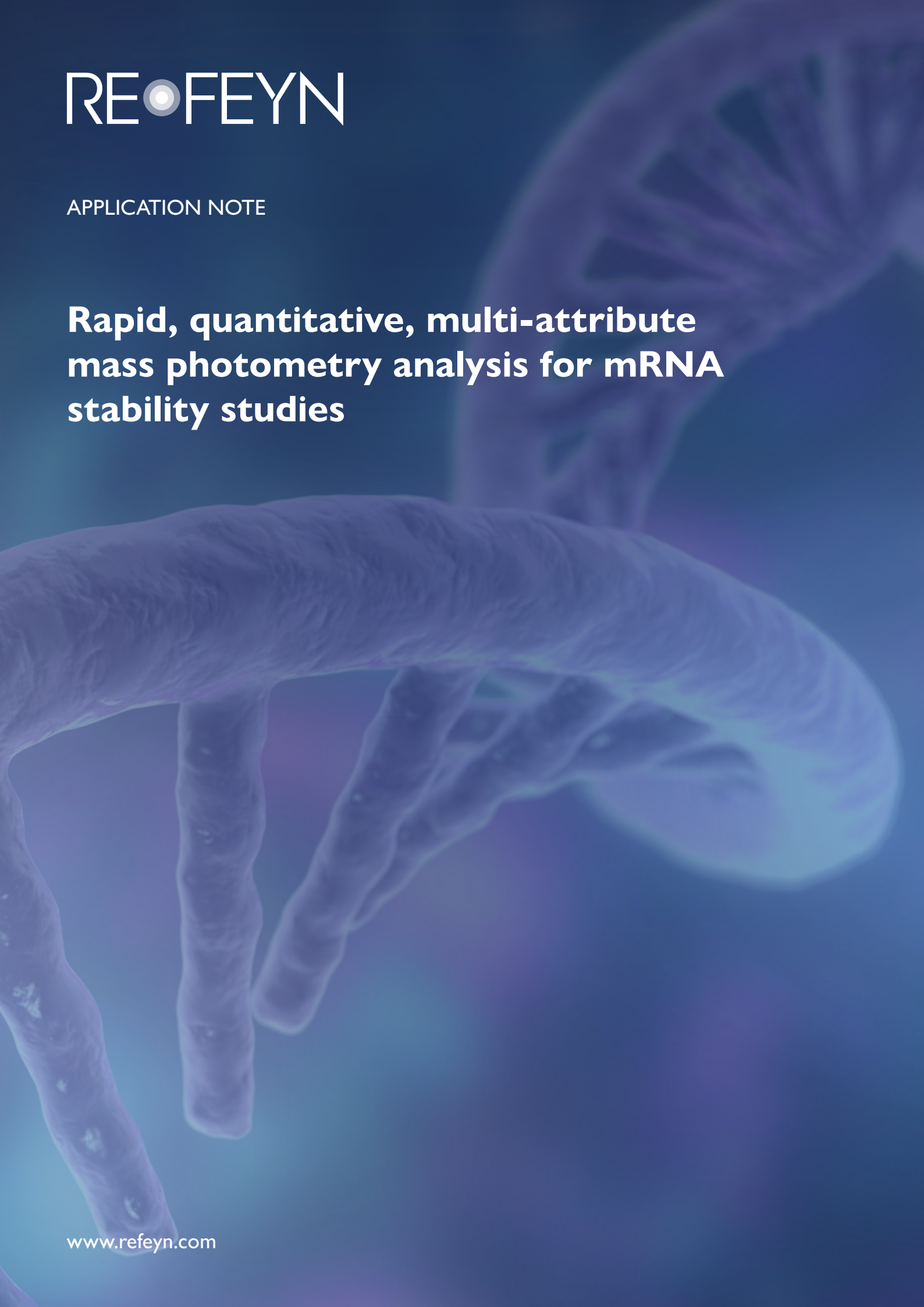
APPLICATION NOTE

Rapid, quantitative, multi-attribute mass photometry analysis for mRNA stability studies



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Abstract

Mass photometry (MP) enables rapid, high-resolution analysis of mRNA integrity and purity under native conditions. It provides single-molecule, quantitative measurements of full-length transcripts, degraded fragments, and aggregates within minutes. Here, three commercial GFP mRNAs were assessed under native conditions, during thermal refolding and denaturation, and during forced degradation. MP revealed differences in sample stability, tracked stress-induced changes, and detected low-abundance species with higher accuracy than CGE. By delivering comprehensive, multi-attribute data in a single instrument, MP streamlines analytical workflows, accelerates development, and supports robust quality assessment of next-generation mRNA therapeutics.

At a glance

- **Rapid and comprehensive analysis:** MP provides a complete assessment of mRNA integrity and native purity in minutes, detecting all species – including full-length transcripts, degradation products, and aggregates.
- **Single-instrument solution:** By directly measuring the mass of individual molecules, MP offers a unified, single-instrument solution to assess mRNA CQAs.
- **Outperforms legacy methods:** MP avoids lengthy run times and complex workflows, and has high accuracy, so it provides faster, more reliable analysis than HPLC and CGE.
- **Versatile applications:** Demonstrated through thermal denaturation/refolding, and forced degradation studies.
- **Accelerates mRNA development:** The speed, accuracy, and completeness of MP enable more efficient workflows, supporting rapid optimization of workflows.

Introduction

Messenger RNA (mRNA) has become central to modern medicine, from vaccines to gene editing and cancer therapies. As the field grows, robust, rapid analytical methods are critical for ensuring quality, safety, and efficacy. Current evaluation of mRNA critical quality attributes (CQAs), such as integrity and purity, relies heavily on legacy techniques, like high-performance liquid chromatography (HPLC) and capillary gel electrophoresis (CGE). While valuable, these methods often have long run times, involve complex data analysis, and exhibit run-to-run variability. Such complications create bottlenecks in development and manufacturing workflows. Furthermore, multi-instrument workflows are incompatible with the pace of modern biopharma.

A central challenge in mRNA analytics is the molecule's inherent instability. mRNA is prone to degradation through multiple routes – including fragmentation during freeze-thaw cycles, structural disruption or misfolding during thermal refolding, and accelerated decay under forced degradation conditions like heating. These stressors reduce the proportion of intact, full-length mRNA, and can generate heterogeneous mixtures of truncated species and aggregates. Such degradation complicates downstream analyses and masks true integrity, underscoring the need for techniques that can sensitively and rapidly track changes under native conditions.

Mass photometry (MP) is a new technique that provides a unified, single-instrument solution to overcome these challenges. It directly measures the mass (i.e. length) of individual mRNA molecules in their native state, offering a unique window into sample heterogeneity. This enables direct and rapid assessment of sample integrity – by quantifying the percentage of full-length product versus fragments, and sample purity – by detecting aggregates, degradation products, and other impurities under native (non-denaturing) conditions. Reporting multiple CQAs in minutes, MP streamlines analytical workflows and accelerates next-generation mRNA therapeutics development.

In this application note, we demonstrate how MP can track mRNA integrity during thermal refolding and forced degradation, highlighting its utility across a range of stability challenges. Using three commercial GFP mRNAs, we show how MP can assess mRNA CQAs, helping optimize refolding protocols and better understand the behavior of mRNA under stress. We also show how MP provides greater accuracy than traditional techniques, such as CGE.

Baseline measurements under native conditions

We first measured our three samples of GFP mRNA under native conditions in 1X PBS – using both MP and CGE (Fig. 1). The three mRNAs range in length from 750 to 1179 bases with different arrangements of untranslated features (i.e., 5' UTR, 3' UTR, polyA tail) around the GFP coding sequence. Two of the mRNA molecules, from Suppliers A (1179 b) and B (980 b), reportedly use the same codon-optimized GFP sequence from *Aequorea victoria* while the Supplier C mRNA (750 b) uses an engineered version of the coding sequence.

All of the MP profiles (Fig. 1A, Table 1) show three main features: A dominant peak for a species consistent with the expected length of the intact monomer, an array of species – likely degradation products – appearing to the left of the main peak, and aggregate species to the right of the intact monomer. For all three samples, according to MP, the aggregate species have masses indicating they are dimers, while a trimer species is also visible for the Supplier B sample.

The CGE experiments showed similar overall profiles (Fig. 1B, Table 1), but contributions from smaller RNA impurities were less apparent than in MP. A likely reason for this discrepancy between the methods is that low-abundance species can be masked in the baseline when using a fluorescent reporter, as in CGE.

When measuring the length of the mRNA for these three samples, MP was significantly more accurate than CGE (Table 1). For MP, the average relative error was 1.2 +/- 1.0%. For CGE, it was 12.7 +/- 20.1%. Both values are consistent with the error ranges reported by the instrument manufacturers (5% for MP and 30% for CGE).

As expected from the differences in species profiles from MP and CGE, the two methods also returned slightly different results when quantifying the abundances of the different species present. The comparison is based on the percentage weight of each species relative to the total weight of all the species in the sample. Although MP data are typically reported as counts per species, we also present relative abundance based on % weight (calculated as the product of the counts of each species

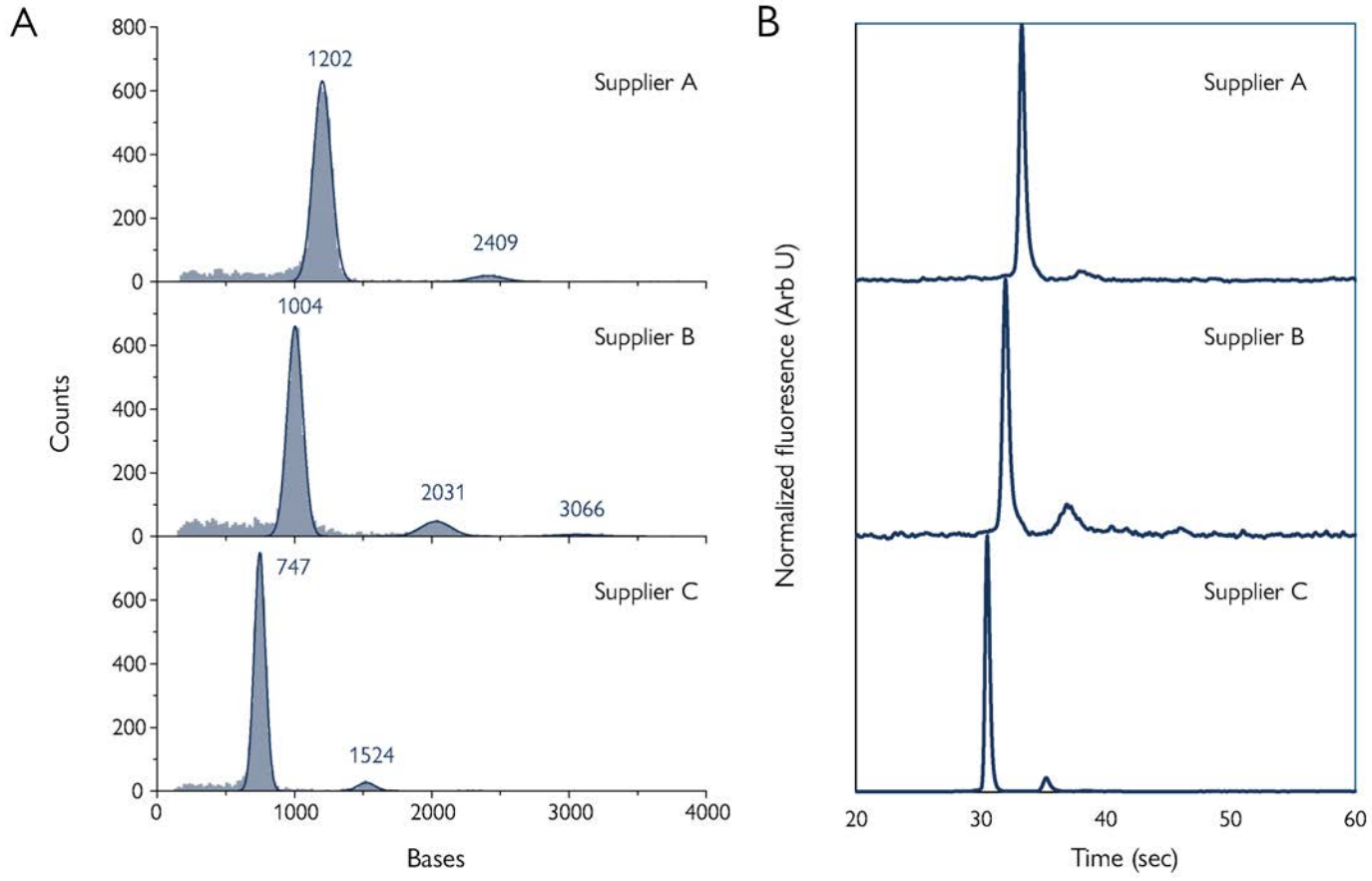

Figure 1 Comparative MP and CGE analysis of GFP mRNAs from different commercial suppliers under native conditions. Samples from three suppliers were analyzed by MP **(A)** and CGE **(B)**. Samples were prepared in 1x PBS at 5 nM. For MP, experiments were done in triplicate (representative measurements shown here) and the labels indicate the mean mass values for each peak. For CGE, there were single measurements.

and its molecular mass, normalized by the total mass of all detected species). This mass-weighted representation of the MP data enables more direct comparison with CGE outputs.

In terms of differences between the methods, for the sample from Supplier A for example, MP reported that 11% of the species were degradation products, 72% monomers and 9% dimers; CGE, meanwhile, reported 5% degradation products, 87% monomers and 9% dimers (Table 1). While the true species abundances in these samples are unknown, MP's greater accuracy for the length measurements supports confidence in its measurements.

Based on the data from MP, there were pronounced differences in monomer abundance across the GFP samples under native conditions. The mRNA from Supplier B showed 52% intact monomer, which is considerably less than in the samples from Supplier A (72%) and Supplier C (76%).

Overall, these data show that MP length measurements are more accurate than those from CGE. This greater accuracy may also extend to measures of the relative abundance of different species, as MP's single-molecule resolution provides a more complete representation of the sample composition. In addition, MP does not require labelling or a secondary reporter, which could mask the visibility of some low-abundance species.

Table 1. Quantification of MP and CGE analyses of GFP mRNAs from different suppliers under native conditions. Quantification of the data shown in Fig. 1. Expected lengths are based on the manufacturers' data, with the degradation cutoff chosen as a reasonable estimate based on the observed width of the monomer peak. For the MP data, relative species abundance by number (% count) was calculated as the counts in the species mass range (using a Gaussian fit to the peak) divided by the total counts in the sample; relative species mass (% weight) was determined from multiplying the % count by the mass for each species – and then expressing this value relative to the total mass of all the species detected. For the CGE data, relative species mass (% weight) was determined from the relative area under the curve. MP measurements were performed in triplicate; CGE measurements were single experiments. Percentages do not sum to 100 because aggregates larger than dimers are not included, and due to rounding.

			Mass Photometry				Capillary Gel Electrophoresis		
Supplier	Species type	Expected length (b)	Measured length (b)	Rel. error (% length)	Species (% counts)	Species (% weight)	Measured length (b)	Rel. error (% length)	Species (% weight)
A	Degradation	<1100	--	--	26	11	--	--	5
	Monomer	1,179	1,199	1.7	67	72	1,399	18.7	87
	Dimer	2,358	2,418	2.5	4	9	2,782	18.0	9
B	Degradation	<900	--	--	20	8	--	--	7
	Monomer	980	982	0.2	62	52	1,017	3.8	64
	Dimer	1,960	1,985	1.3	9	23	2,502	27.7	20
C	Degradation	<700	--	--	9	4	--	--	2
	Monomer	750	750	0.0	81	76	576	-23.2	89
	Dimer	1,500	1,523	1.5	6	10	1,973	31.5	7

Thermal denaturation and refolding

The thermal denaturation and refolding of mRNA is a critical and straightforward technique used to ensure the functional integrity of transcripts. While the codon sequence of an mRNA dictates the protein it will encode, internal base pairing gives rise to secondary and tertiary structures that are equally vital for its proper function within a cell. Following synthesis, mRNA molecules can misfold into undesirable structures that hinder crucial processes, like translation, or lead to aggregation. To resolve this complication, a brief heating step is often applied to denature or 'melt' these misfolded structures, followed by a controlled cooling process to promote the refolding of the mRNA into a more functional conformation. This simple but powerful method is a fundamental step in optimizing mRNA performance for a variety of applications, from vaccine development to therapeutic protein production.

We used MP to assess how each of the three GFP mRNAs responded to two minutes of heating at 70 °C, 80 °C, or 90 °C (with a sample placed at 4 °C as a control). The mass distributions from Supplier A are shown as an example (Fig. 2), with integrity and purity results from all three samples summarized in Fig. 3. We observed that all three samples, when heated, lost their aggregate structures. As the Supplier A example shows, a small population of dimers (~2,358 b) was visible for the 4 °C control, but not for the heated samples. All three samples also showed an accumulation of low-mass degradation products after heating (visible to the left of the monomer peak), and it was most apparent for the Supplier A sample (Fig. 2).

The MP analysis enabled us to assess the integrity and purity of each mRNA after exposure to the controlled thermal stress (Fig. 3). In terms of integrity, for every sample, the monomer length remained within 4% of the expected length, which is within the instrument's error margins – indicating that integrity was not significantly affected by the thermal stress (Fig. 3A). For sample purity (the percentage of intact mRNA monomer present), there were more noticeable changes, and each mRNA

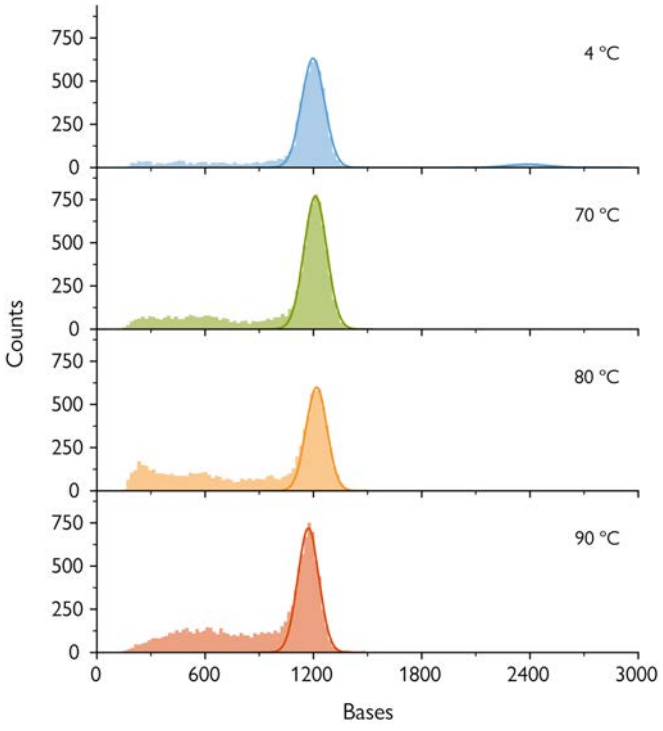

Figure 2 Representative MP data for thermal denaturation and refolding of mRNA from Supplier A. Samples from Supplier A (0.1 mg/mL) were incubated at 4 °C (blue), 70 °C (green), 80 °C (orange), or 90 °C (red) for 2 min before being placed on ice. Samples were then diluted 1:20 in 1x PBS for analysis. For MP, experiments were done in triplicate (representative measurements shown here).

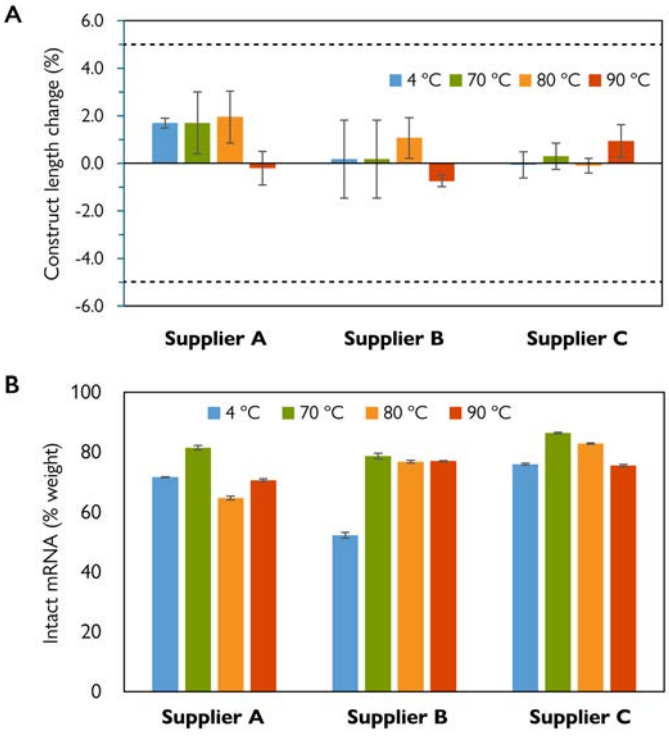

Figure 3 Impact of thermal denaturation and refolding on GFP mRNA integrity and purity, as determined by MP. Integrity, as measured by changes in construct length **(A)**, and purity, as measured by the relative abundance of full-length mRNA monomers **(B)** were assessed by MP for three commercial mRNAs after a two-minute incubation at 70 °C, 80 °C, or 90 °C. The 4 °C control was stored on ice. In **(A)**, the dashed lines at ±5% indicate the error margin for the TwoMP mass photometer used for the analysis. All data represent the mean ± standard deviation from triplicate measurements of each sample.

had a slightly different response to thermal refolding (Fig. 3B). In all three samples, integrity was highest at 70 °C, then decreased to varying degrees at 80 °C and appeared to increase slightly (Supplier A), remain about the same (B) or decrease again (C) at 90 °C. All samples were also quantified by UV-Vis and showed consistent values, indicating that no material was lost to precipitation or to the formation of aggregates large enough to be beyond the MP detection range (data not shown).

Forced degradation under high heat stress

Studying mRNA degradation under forced heating conditions offers significant advantages for biopharma as it accelerates the degradation process, providing a rapid way to simulate long-term stability. By subjecting the mRNA to high temperatures, researchers can quickly identify degradation pathways and sensitive sequences within the transcript. Such data are crucial for developing a stable formulation, as it helps guide the selection of excipients and buffer systems that can protect the mRNA from thermal stress. Ultimately, understanding how an mRNA transcript degrades under forced conditions is essential for developing a robust, stable product that maintains its integrity and efficacy throughout its intended shelf life.

We used MP to assess how the three commercial GFP mRNA samples responded to forced degradation – exposure to 90 °C heat – with measurements at regular intervals over a period of 8 minutes. Degraded mRNA products are most notably observed as lower-mass species. The three GFP mRNAs behaved broadly similarly, with the main peak decreasing in height as degradation products (species with lower mass than the monomer) accumulated (Fig. 4). As observed in the thermal denaturation and refolding experiments, there were aggregates present initially (Fig. 4, insets), but they were depleted after the initial minute of heating. By 8 minutes of exposure to 90 °C heat, all 3 samples showed a significant loss of intact mRNA, with degradation products becoming more abundant than intact monomers (Fig. 4).

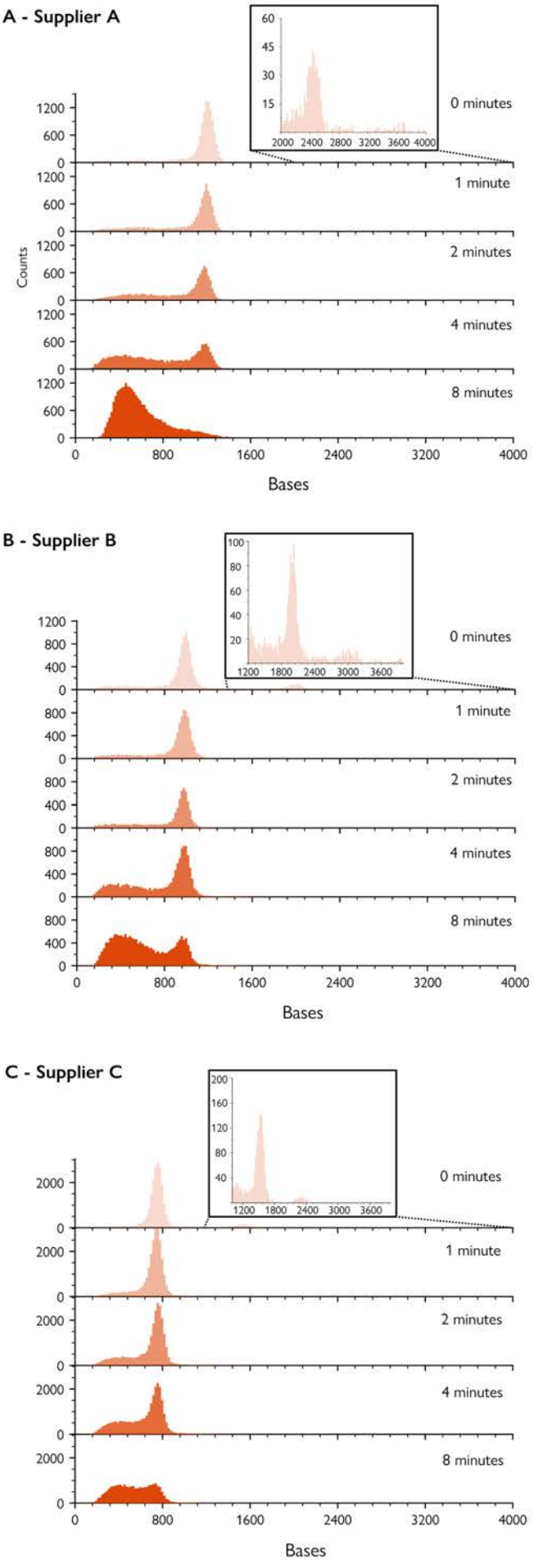

Figure 4 Mass distributions for commercial GFP mRNA samples undergoing forced heat degradation (90 °C), as measured by MP. Samples from Suppliers A **(A)**, B **(B)**, and C **(C)**, each at a concentration of 0.1 mg/mL, were heated at 90 °C for 0 min, 1 min, 2 min, 4 min, or 8 min. The graphs are shaded red, and are lightest for the 0 min sample and then become darker for each time point.

The measured length of the intact mRNA monomer also broadly decreased over time at 90 °C for all three samples (Fig. 5A), although the measured length of the monomer remained within the instrument's error range (±5%) relative to the 4 °C control throughout the 8 minutes for the samples from Suppliers B and C.

The breakdown of aggregates and degradation of monomers were also apparent from quantification of the abundance of each species over time (Fig. 5B). Over time, all three samples showed an initial increase in the abundance of monomers, likely due to a breakdown of aggregates – suggesting that 1 minute of heating at 90 °C was enough to break apart most aggregates. Next, they showed a clear decline in the abundance of monomers. The mRNA from Supplier C retained the highest level of intact monomer after heating, possibly due to being an engineered sequence.

Conclusion

In the rapidly advancing field of mRNA therapeutics, there is a pressing need for robust, rapid analytical methods to ensure product quality. Traditional legacy techniques, such as HPLC and CGE, often create analytical bottlenecks due to lengthy run times or sample preparation, complex workflows, and limited accuracy. Overall, these methods cannot be relied on to quickly provide the comprehensive view of sample heterogeneity that is needed to support mRNA therapeutics development. This application note has shown how MP serves as a next-generation analytical technique that overcomes these challenges by providing a complete, quantitative analysis of mRNA integrity and native purity. Within minutes, mass photometry reveals and quantifies all species present in the sample, including full-length transcripts, degraded species, and aggregates.

Here, we demonstrated these capabilities through experiments exploring thermal denaturation and refolding, and forced degradation. The results highlight MP's ability to directly quantify mRNA integrity under stress, providing crucial insights into how mRNA design and formulation influence overall stability. Ultimately, the speed, accuracy, and comprehensive nature of MP data enable a more streamlined and efficient workflow for mRNA development, accelerating the path from research to therapy.

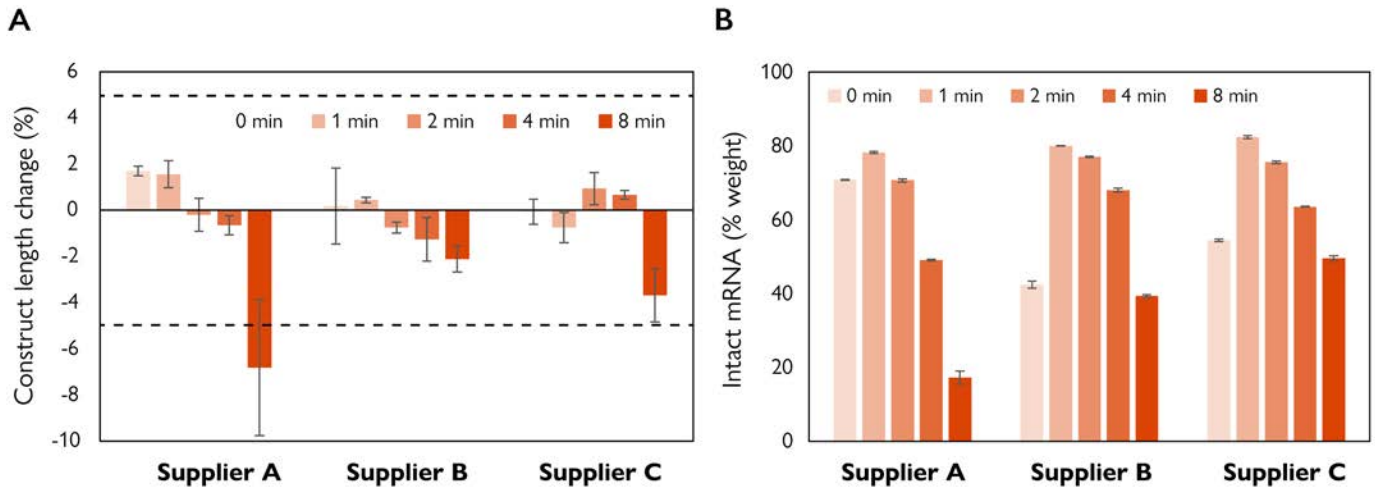

Figure 5 Impact of forced degradation on GFP mRNA integrity and purity, as determined by MP. Integrity, as measured by changes in construct length **(A)**, and purity, as measured by the relative abundance of full-length mRNA monomers **(B)** were assessed by MP for three commercial mRNAs during exposure to 90 °C, with regular measurements over 8 minutes. In **(A),** the dashed lines at ±5% indicate the error margin for the TwoMP mass photometer used for the analysis. All data represent the mean ± standard deviation from triplicate measurements of each sample.

Materials and methods

Reagents

GFP mRNA samples were sourced from PackGene (Houston, TX, USA – Supplier A), GenScript (Piscataway, NJ, USA – Supplier B), and Aldevron (Madison, WI, USA – Supplier C). All are reportedly chemically capped and polyadenylated. Nuclease-free water (New England Biolabs, B11500S); Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, 14190144). MP experiments were performed with MassGlass™ NA slides (Refeyn, MP-CON-21024).

Mass photometry

Instrument: TwoMP mass photometer (Refeyn Ltd., Oxford, UK). Regular calibration was done using Millennium RNA markers (ThermoFisher, AM7150) at a 1:200 dilution in 1x PBS. Prior to each measurement, instrument focus was stabilized on buffer to minimize background scattering. A 10 µL droplet of buffer was used to focus the instrument before loading a 10 µL mRNA sample (5 ng/µL) into the imaging well. Movies were recorded for 60 sec using regular mode and a large field of view in the AcquireMP software. Events corresponding to individual mRNA molecules binding to the glass–solution interface were detected and analyzed using Refeyn DiscoverMP software (v2025 R1).

Capillary gel electrophoresis

Instrument: Agilent 2100 BioAnalyzer with picoRNA assay kit (Agilent Technologies, Cat. No. 5067-1513) according to the manufacturer's instructions. Briefly, 1 µL of each mRNA sample (5 ng/µL) was mixed with the supplied gel–dye solution and loaded onto an RNA pico chip prefilled with gel matrix, along with the RNA ladder (200–6000 nt) as a size reference. CGE experiments were run under the default program using the Agilent 2100 Expert software (vB.02.12.2420).

mRNA refolding

Thermal denaturation and refolding experiments were done on all three mRNAs to compare the refolding response of each sample. Samples were prepared at 0.1 mg/mL in nuclease-free water and split into four aliquots for the experiment. One sample was set on ice as a control while the other three samples were heated for two minutes at 70 °C, 80 °C, or 90 °C using a thermal cycler. Immediately after heating, samples were rapidly cooled to 4 °C and held for 5 min to enable refolding of the mRNA structure. All samples were then diluted 1:20 with 1x PBS before analysis.

Forced degradation of mRNA at 90 °C

Forced degradation experiments were done on all three mRNAs to better understand the sensitivity of these reagents to overheating relative to the refolding experiments shown above. Samples were prepared at 0.1 mg/mL in nuclease-free water and split into aliquots for each time point at 90 °C. One aliquot of each mRNA was set on ice as a control. Immediately after heating, samples were rapidly cooled to 4 °C to promote intramolecular refolding and held at 4 °C for 5 min to allow the sample to reach equilibrium. All samples were then diluted 1:20 with 1x PBS before analysis.

UV-Vis

Sample concentrations were determined by UV-Vis spectroscopy using a Nanodrop Lite Plus (ThermoFisher).

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