Novel Mobile Phase to Control Charge States and Metal Adducts in the LC/MS for mRNA Characterization Assays

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ABSTRACT: Mass spectrometry is a widely used tool in the characterization of oligonucleotides. This analysis can be challenging due to the large number of possible charge states of oligonucleotides, which can limit the sensitivity of the assay, along with the propensity of oligonucleotides to readily form adducts with free alkali metals. To reduce the adduct formation, oligonucleotides are typically purified with desalting columns prior to analysis. We have developed a mobile phase that gives superior reduction in charge states and adduct formation compared to previously reported methods and, more importantly, obviates the requirement of desalting samples prior to mass spectrometric analysis, significantly decreasing the sample preparation time and amount of RNA required for analysis. We have applied this mobile phase to develop methods to quantify the 5′-capping efficiency and to characterize the polyadenosine (poly(A)) tail of mRNA synthesized in vitro: two critical quality attributes of mRNA therapeutics. Through this, we were able to demonstrate RNA that was co-transcriptionally capped to have capping efficiency equivalent (the percent total molecules that contain a cap) to other reports in the literature using materials that were generated using the same synthesis procedure. Furthermore, by using a mobile phase mixture comprised of hexafluoroisopropanol, triethylammonium acetate, triethylamine, and ethanol, we were able to determine the size distribution of the poly(A) tail in various mRNA samples from DNA templates that ranged from 50 to 150 nt poly(A) and verify that distribution with commercially available RNA standards, successfully demonstrating that this mobile phase composition could be used for characterization assays for both mRNA caps and tails.

■ INTRODUCTION

Over the last 30 years, there has been a steady increase in interest in the field of messenger RNA (mRNA) therapeutics. The first report of protein expression from exogenous/synthesized mRNA in an animal model was published in 1990.1 It was quickly recognized that the stability of mRNA was a major challenge to the development of mRNA therapeutics,2 and R&D effort was instead spent studying DNA and protein therapeutics.2,3 However, significant improvements in stability and design of exogenous/synthesized mRNA have been made over the last 10 years. The development of enzymatic and co-transcriptional mRNA capping methods has allowed for the production of mRNA capable of higher rates of translation in eukaryotic systems. And the development of DNA templates for in vitro transcription has allowed for the production of molecules with longer and more homogeneous distributions of poly(A) tails, a feature of mRNA that influences translation.4 These advances have spurred progress with mRNA therapeutics; to date, the FDA has authorized the emergency use of two mRNA vaccines one of which just received full approval, both for COVID-19.5 To improve the oligonucleotide process development, analytical methods are needed to characterize the mRNA caps and the poly(A) tail as both attributes are critical for the successful translation of proteins.

The 5′-cap is required for cellular functions in eukaryotes. The 5′-cap comprises an inverted 7-methylguanosine connected to the first nucleotide of the mRNA by a 5′-5′ triphosphate bridge.

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a structure known as Cap 0. For many endogenous mRNAs in eukaryotes, the second nucleotide could have a 2′-O-methylation, a structure known as Cap 1. Further methylation is possible and would be called Cap x, where x stands for the number of the consecutive methylation. These features are known to have interactions with binding proteins and complexes, which are required for RNA processing and translation. The cap also protects transcripts from degradation and cap regulation can change gene expression and cellular function. For both co-transcriptionally and enzymatically generated 5′-mRNA caps, there have been three primary assays to characterize the capping efficiency. The first involves annealing a DNA oligonucleotide to the mRNA, forming an RNA–DNA complex, selectively cleaving the 5′-side off the intact RNA, and determining the capping percentage by high-resolution mass spectrometry. For the second and third assays, intact 32P labeled or unlabeled RNA can be digested using nonspecific RNases, resulting in mRNA fragments with 5′ caps that can be quantified using high-pressure liquid chromatography (HPLC), thin-layer chromatography (TLC), or agarose gels. RNA can be capped either enzymatically post-synthesis or co-transcriptionally during the synthesis of the RNA capping, in which an analog containing the 5′-mRNA cap can be directly incorporated into the product during synthesis. There have been reports of greater than 90% capped RNA using the analog during in vitro transcription reactions. Alternatively, RNA can be capped post-synthesis using enzymes that can now be expressed in industrially relevant Escherichia coli strains such as the vaccinia capping enzyme. This enzyme is composed of two subunits that contain three enzymatic activities: (1) RNA triphosphatase, (2) guanyltransferase, and (3) guanine methyltransferase, all of which are necessary for the addition of a cap structure.

It is important to have an intact poly(A) tail to prevent degradation of the mRNA prior to translation. The 3′ poly(A) tail primarily acts as a stabilizer of intact mRNA in eukaryotes by preventing enzymatic degradation and enhancing translation efficiency. The degradation of mRNA is typically determined by poly(A) shortening by a deadenylase and is the limiting factor in mRNA stability. Once the poly(A) tail is removed, the mRNA cap can immediately be decapped, stopping translation. Commonly, poly(A) tails are incorporated into the DNA templates for mRNA synthesis either as a sequence cloned into a plasmid or added by PCR amplification. Alternatively, a poly(A) tail can also be added post-transcriptionally using poly(A) polymerase. Methods exist that use precision digestion, using RNase H or RNase T1, to cleave the poly(A) tail fragment from the full sequence, which are further isolated and concentrated with oligo dT purification. The resulting fragments are then used for analysis often by mass spectrometry or agarose gels.

Mass spectrometry has become the gold standard for characterizing oligonucleotides. The ability to obtain structural, sequence, and quantitative information as well as intact mass of the sequences sets it apart from many of the other available techniques. Oligonucleotides are primarily ionized using negative-ion electrospray ionization (ESI) and a mixture of triethylamine (TEA) and 1,1,1,3,3,3-hexafluoro-2-propanol, acetonitrile, ethanol (LC/MS grade), formic acid, methanol (LC/MS grade), triethylamine, triethylammonium acetate, phenol:chloroform, water (LC/MS grade), and isopropanol were all obtained from Sigma-Aldrich (MO). Custom oligonucleotides were obtained from IDT. Custom poly(A) standards were obtained from the Horizon Discovery (CO) DNApAc RP 4 μm × 2.1 mm × 100 mm HPLC column, 7.5 M LiCl solution, nuclease-free water, RNase T1, DNase I, PCR strip tubes, and glass HPLC vials with caps were all obtained from Thermofisher Scientific (NJ). RNase H, RNase H buffer, Cutsmart buffer, and HiScribeT7 High Yield RNA Synthesis Kit were obtained from New England Biolabs (MA). QIAprep Miniprep kit was obtained from Qiagen (MD). CleanCap Reagent AG and CleanCap Reagent AG (3′ OMe) were purchased from TriLink Biotechnologies (CA).

2. MATERIALS AND METHODS

2.1. Chemicals and Materials. 1,1,1,3,3,3-Hexafluoro-2-propanol, acetonitrile, ethanol (LC/MS grade), formic acid, methanol (LC/MS grade), triethylamine, triethylammonium acetate, phenol:chloroform, water (LC/MS grade), and isopropanol were all obtained from Sigma-Aldrich (MO). Custom oligonucleotides were obtained from IDT. Custom poly(A) standards were obtained from the Horizon Discovery (CO) DNApAc RP 4 μm × 2.1 mm × 100 mm HPLC column, 7.5 M LiCl solution, nuclease-free water, RNase T1, DNase I, PCR strip tubes, and glass HPLC vials with caps were all obtained from Thermofisher Scientific (NJ). RNase H, RNase H buffer, Cutsmart buffer, and HiScribeT7 High Yield RNA Synthesis Kit were obtained from New England Biolabs (MA). QIAprep Miniprep kit was obtained from Qiagen (MD). CleanCap Reagent AG and CleanCap Reagent AG (3′ OMe) were purchased from TriLink Biotechnologies (CA).

2.2. Capping Assay Calibration Curve Preparation. Capped mRNA was synthesized using the CleanCap protocol reported by Henderson et al. The mRNA was purified using 1 volume sample to 1.5 volumes of 7.5 M LiCl and incubated at −30 °C for 30 min. The sample was centrifuged at 1792g at 4 °C for 15 min to pellet the RNA. The supernatant was removed and rinsed with 2 volumes of 70% ethanol. The sample was air-dried for 10 min. The sample was resuspended in 1 volume of nuclease-free water. An aliquot of the stock solution was diluted and measured using UV absorbance at 260 nm to determine the concentration of the solution. An additional aliquot was diluted 10-fold and 2 μL was injected by HPLC for purity determination.

2.3. Capping Assay Sample Preparation. To PCR tubes, 50 μL of the RNA sample (10 pmol), 12 μL of RNase H buffer, 2.5 μL of cleavage probe (50 pmol), and 1 μL of internal sequence, and quantitative information as well as intact mass of the sequences sets it apart from many of the other available techniques. Oligonucleotides are primarily ionized using negative-ion electrospray ionization (ESI) and a mixture of triethylamine (TEA) and 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) in the HPLC mobile phases. The unique properties of HFIP, which can adjust the pH of the solution and also enhance the ionization of the oligonucleotide, are the reasons for its frequent use. Other groups have been studying the effects of other organic modifiers and one of the major findings is that the volatility of the alkylamine has an important role in the charge state distribution and desorption efficiency of the oligonucleotide. Additionally, the organic eluant in the mobile phase is equally important. For example, HFIP has low solubility in acetonitrile, so methanol is commonly used for this ion pair reagent. One of the limitations of mass spectrometry for oligonucleotides is the amount of charge states that are observed due to the different interactions on the phosphate backbone.

Current analytical columns do not have the resolution to resolve N-1 species for RNA larger than about 90 nucleotides and the resolution of the mass spectrometer is the limiting factor to accurately deconvolute the oligonucleotide. If the charge states can be reduced, the mass-to-charge ratio of the ions will be higher. For larger RNA, where baseline separation of different lengths by an HPLC column is more difficult, it then becomes easier to deconvolute a mixture of oligonucleotides.

In this study, the aim was to develop a mobile phase blend that could reduce the number of charge states and adduct formations for oligonucleotides by mass spectrometry and use this mobile phase blend to develop robust methods to characterize the cap and tail structures of mRNA. By using a mixture of 30 mM HFIP, 10 mM TEAA and 1.2 mM TEA, and ethanol in our eluent, we were able to develop poly(A) tail and capping efficiency assays that required no additional sample preparation post-digestion. The cap assay could be performed with as little as 30 pmol and the poly(A) tail assay with as little as 50 pmol RNA.
standard (5 pmol) were added and mixed well by inversion. In a thermocycler, the samples were heated to 95 °C for 5 min, followed by 65 °C for 2 min, 55 °C for 2 min, 40 °C for 2 min, and finally 22 °C for 2 min. After the annealing cycle, 5 μL of RNase H was added to the tubes and mixed well by inversion. The samples were heated for 2 h at 37 °C and transferred to HPLC vials for analysis afterward. The internal standard is a representative hydroxylated 5′-RNA that anneals to the same DNA probe and is used to monitor digestion efficiency in the samples.

2.4. Capping Assay Analytical Method. Samples were analyzed by reversed-phase ion pair chromatography on a Vanquish UHPLC using a DNA Pac RP 4 μm × 100 mm × 2.1 mm ID column connected to a Q Exactive Plus orbitrap mass spectrometer from Thermo Scientific (NY). Mobile phase A consisted of 30 mM HFIP, 10 mM TEAA, and 1.2 mM TEA. Mobile Phase B consisted of 10 mM TEAA in 50% ethanol. The column temperature was held constant at 65 °C with a flow rate of 0.4 mL/min. The chromatographic separation was performed using the following gradient: starting at 1% buffer B and held for 0.7 min, followed by a ramp to 35% buffer B over 4.3 min, a ramp to 80% buffer B over 1 min, a hold at 80% buffer B for 1.5 min, and an immediate return to 1% buffer B and held for 2.5 min. All mass spectra were obtained in the negative-ion mode over a scan range of 1150–2500 m/z with a resolution of 70,000. Source and capillary temperatures were set to 350 °C and all spectra were analyzed using BioPharma Finder from Thermo Scientific with deconvolution parameters shown in Figure S2 (NY).

2.5. Poly(A) Tail Assay Sample Preparation. To PCR tubes, 50 μL of the RNA sample (at least 47 pmol), 5 μL of Cutsmart buffer, and 1.6 μL of RNase T1 (50 U/μL) were added and mixed well by inversion. The samples were heated for 2 h at 37 °C and transferred to HPLC vials for analysis afterward.

2.6. Poly(A) Tail Assay Analytical Method. Samples were analyzed by reversed-phase ion pair chromatography on a Vanquish UHPLC using a DNA Pac RP 4 μm × 100 mm × 2.1 mm ID column connected to a Q Exactive Plus orbitrap mass spectrometer from Thermo Scientific (NY). Mobile phase A consisted of 30 mM HFIP, 10 mM TEAA, and 1.2 mM TEA. Mobile Phase B consisted of 20 mM TEAA in 50% ethanol. The column temperature was held constant at 65 °C with a flow rate of 0.4 mL/min. The chromatographic separation was performed using the following gradient: starting at 1% buffer B and held for 0.7 min, followed by a ramp to 35% buffer B over 24.3 min, a ramp to 80% buffer B over 1 min, a hold at 80% buffer B for 1.5 min, and an immediate return to 1% buffer B and held for 2.5 min. All mass spectra were obtained in the negative-ion mode over a scan range of 750–1850 m/z with a resolution of 17,500. The scanning was kept low to increase the sensitivity of the analysis. Source and capillary temperatures were set to 350 °C, and all spectra were analyzed using BioPharma Finder from Thermo Scientific with deconvolution parameters shown in Figure S3 (NY).

2.7. Generation of DNA Templates. Plasmid DNA templates were created by cloning synthetic Poly(A) sequences as annealed complementary oligonucleotides into vectors containing a T7 promoter and a GFP mRNA sequence. These plasmids were isolated using a QIAprep Midiprep kit, linearized by restriction digestion, and purified by phenol chloroform extraction and isopropanol precipitation.

2.8. Diagnostic Restriction Digest to Determine the Length of Poly(A) DNA. Plasmid DNA templates were digested with BspQI and BsrDI from New England Biolabs (Ipswich, MA) to yield three DNA fragments: two high-molecular-weight fragments and one lower-molecular-weight fragment containing the poly(A) sequence. These sizes of the poly(A) containing fragments were determined by capillary electrophoresis using Agilent’s D1000 TapeStation kit and the standard ladder as described by the vendor’s protocol (CA).

2.9. Synthesis of RNA. In vitro transcription was performed using the HiScribeT High Yield RNA Synthesis Kit and CleanCap Reagent AG or CleanCap Reagent AG (5′ OMe). The final concentration of components used was 0.5X reaction buffer, 5 mM each of NTPs, 4 mM CleanCap AG, 30 ng/μL DNA template, and T7 Polymerase Mix as recommended by the manufacturer. The reaction was incubated for 2 h at 37 °C. The reaction was treated with DNase I, 0.1 U/μg DNA, heated for 30 min at 37 °C, and purified by precipitation with 7.5 M LiCl. RNA quality (A260/A230 ratio) and concentration were measured spectrophotometrically.

3. RESULTS AND DISCUSSION

3.1. Mobile Phase Optimization. We aimed to create an improved analytical method that could characterize the 5′ cap and poly(A) tail components of our in-house synthesized mRNA. To achieve this, the primary focus was to develop methods that could detect oligonucleotides between 20 and 200 nt. Currently, the most common way to characterize the cap and the tail is through methods that utilize the mobile phase containing a mixture of HFIP and TEA. Our experience with this mobile phase blend led us to inconsistent results that often contained substantial sodium adducts despite utilizing all combinations of system cleaning, mass spectrometer (MS) tuning, and use of different reagent vendors with the highest grade solvents available. For mRNA cap analysis, this was not particularly a problem because the smaller fragments can easily be separated by HPLC. However, for poly(A) tail characterization, the larger sizes are not separated by HPLC, and due to the multiple species and multiple salt adducts, we were unable to deconvolute the intact masses. We had explored other modes of separation including HILIC and reversed-phase (without ion pairing) chromatography, but we were unable to achieve sufficient sensitivity compared to the traditional HFIP mobile phase mixture. We started optimization with 400 mM HFIP, 12 mM TEA using methanol as the eluent and quickly realized that there was no benefit from using buffer concentrations so high and saw similar performance after reduction of the buffers four-fold. However, we were unable to control the amount of sodium adducts that appeared at varying levels in our samples. We explored TEAA and observed similar charge state reductions as previously reported.23−25 Though for concentrations above 10 mM TEAA, we observed a significant loss of signal. This initial work showed promising results with TEAA alone, which worked well for the mRNA caps, but was not sensitive enough to detect the poly(A) tail distribution. We wanted to evaluate the findings reported by Chen et al. and test other organic solvents, particularly ethanol in our mobile phases.23 What we found matched their reports and we were able to shift the oligonucleotides to lower charge states and reduce the amount of adducts in our samples. Even with ethanol or isopropanol as the eluent, we observed about a 50% reduction in ion intensity. This led us to explore mixtures of multiple ion pair reagents and found that using combinations of HFIP and TEA to maximize sensitivity. We found an optimal result with a mixture of 30 mM HFIP and 1.2 mM TEA but increasing the...
A 22-mer RNA oligonucleotide was synthesized, which mimics the cleaved product obtained from the RNase digestion of our test sequence. This oligonucleotide was used to optimize concentration of HFIP/TEA beyond this led to the appearance of a high level of sodium adducts again.

Figure 1. Using different combinations of mobile phases as indicated above each spectrum, a 25 ng/μL 22-mer custom RNA oligonucleotide was analyzed. The x-axis represents the mass-to-charge ratio and the y-axis represents ion intensity in the spectra. (1A) Higher charge states dominate the spectrum with noticeable adducts present. These adducts are easily observed when zooming in on the various m/z’s with charge state 4, as shown in 1B. (2A) There is a marked shift to smaller charge states. (2B) The adduct formation decreased substantially without HFIP which is supported by the decrease in the number of adduct clusters as well as the lower intensity signal of the remaining adducts. (3A) Combination of HFIP and TEAA produces a simpler spectrum with a lower level of adducts than HFIP alone. (3B) The benefit of this mobile phase combination can be further appreciated when compared to the earlier mobile phase blends (1B, 2B) and noting the lack of adducts present in the 1805 m/z range and higher.
the MS method and mobile phase composition. Figure 1 shows the base peak intensity of a 22-mer with three combinations of mobile phase compositions. Using a mobile phase containing 300 mM HFIP and 12 mM TEA alone, higher-order charge states are observed, but the signal is split across multiple charge states. We also observed the highest number of adducts in this mobile phase. When using 20 mM TEAA, fewer adducts and a substantial reduction of charge states above 4 are observed but the ion intensity is 50% lower than the HFIP/TEA mixture. By combining HFIP/TEA and TEAA, the signal is comparable to HFIP and TEA alone, but the charge state and adduct formation are still significantly reduced (Figure 1). It was important to use ethanol, rather than traditional acetonitrile or methanol, or substantial ion suppression was observed, which was previously reported by Weng et al. It is believed that the ethanol improves the evaporation rates for the TEAA containing charged droplets by reducing the activation energy needed for evaporation. For HFIP concentrations higher than 30 mM, the amount of adducts observed was increased.

From this set of experiments, we were able to determine a suitable mobile phase composition—30 mM HFIP, 10 mM TEAA, and 1.2 mM TEA—to yield a simpler spectrum while maintaining ion signal intensity that can be utilized for our assay development. We note that chromatographic resolution was not impacted (positively nor negatively) with this mobile phase; the improvements stem from resolution within the mass spectrometer.

3.2. mRNA Capping Assay Sample Preparation Optimization. Recently, Beverly et al. reported a method to quantify 5'-caps of mRNA. In this method, a complementary biotinylated ssDNA oligonucleotide was annealed to the mRNA on the 5' side to form an RNA/DNA hybrid. Then, RNase H was used to cleave the RNA/DNA hybrid, liberating small 5'-capped fragments of mRNA. The cap-containing RNA can be isolated using magnetic streptavidin beads because the biotinylated cleaved RNA/DNA hybrid can bind to the beads and subsequently be denatured with heat, releasing the cleaved RNA oligonucleotide. The cleaved RNA fragment was dried to concentrate the sample and remove organic solvents and then reconstituted in the mobile phase for analysis. For the reported assay, 100 pmol of RNA is required due to the low recovery of the streptavidin isolation step, which for a 4100 nt sequence would equate to approximately 130 μg. Because of this sample requirement, we wanted to evaluate the feasibility of an approach that not only minimizes the sample needed but also the sample preparation time, as the proposed protocol was labor-intensive.

Initial feasibility tests were performed using two sources of firefly luciferase mRNA. Both were synthesized by in vitro transcription, with one generated in-house using canonical nucleotides and the other synthesized by TriLink Biotechno-
ologies with canonical nucleotides and the co-transcription capping reagent CleanCap AG. Both the capped and uncapped oligonucleotide masses were detected in the deconvoluted spectrum from the corresponding samples (Figure 2).

To determine the sensitivity of the assay, a titration of capped RNA was prepared in triplicate ranging from 0 to 100% of each analyte. The responses for the capped RNA were linear with $R^2 > 0.99$ (Figure S1). We were able to differentiate 2% uncapped mRNA by using only 15 pmol of the material.

### 3.3. Poly(A) Tail Analytical Method Optimization

In the initial method development, we had tried using the HFIP/TEA blend that is commonly used for oligonucleotide analysis but observed a large amount of sodium adducts. (Section 3.1) Attempts to reduce this included optimizing the source conditions, passivating the HPLC system with nitric acid, and adding trace amounts of EDTA into the mobile phases. While we were successful in reducing the adducts, they quickly returned and were not consistent. A 100-mer poly(A) standard synthesized by Horizon was used to evaluate both mobile phases. In the HFIP/TEA mobile phase, the salt adducts dominate the spectrum and the charge states are barely visible (Figure 3A). With the TEAA blend, the charge states are clearly visible with a noticeable reduction in adducts (Figure 3B). Because of this improvement, we decided to move forward with this mobile phase composition for future development.

### 3.4. Poly(A) Tail Method Validation

To evaluate the sensitivity of the assay, a titration of 4100 nt mRNA with an expected poly(A) tail length of 100 nt was prepared at concentrations ranging between 50 and 250 pmol and digested in triplicate. We were unable to detect the poly(A) tail consistently in the 31 pmol samples, so those data are not shown. The distributions for the lowest and highest detectable samples are shown in Figure 4. The x-axis represents the nucleotide length, and the y-axis represents the % signal detected of that specific poly(A) tail length divided by all poly(A) lengths detected. One observation from the titration data was that we detected more distinct lengths at higher RNA load. However, by calculating the weighted average poly(A) tail lengths, we observed no increase in the average length as the concentration of RNA was increased (Figure 5), indicating the lack of bias in the assay with respect to input mass.

A major concern we had from previous methods was the size selection bias introduced from an oligo dT affinity purification of the poly(A) tail. There have been multiple reports of higher recoveries observed from RNA with longer poly(A) tails.20,25,26 By taking a “chop-and-shoot” approach, we should see all poly(A) lengths equally because we will be directly measuring...
the poly(A) tail fragments in the sample without any additional sample cleanup. A sample containing 250 pmol of 4100 nt mRNA was spiked with a custom poly(A) ladder containing synthetic oligonucleotides with 40, 60, and 80 nt lengths. We were able to detect the poly(A) distribution from our sample along with the spiked standards (Figure 6).

To test accuracy, we purchased FLuc mRNA from a commercial vendor (TriLink Biotechnologies), which should have a poly(A) distribution around 120 nt. Three 125 pmol RNA digestions were prepared and analyzed by our method. We calculated an average weighted poly(A) tail length of 123 nt (Figure 7).

To test the utility of our mobile phase to resolve poly(A) species of multiple sizes, we engineered plasmid DNA templates to produce GFP mRNA with poly(A) tails of different lengths: 0, 50, 80, 100, 120, and 150 bp. These plasmids were propagated in E. coli, isolated by Minipreps, and linearized with a BspQI. The lengths of poly(A) tails in these DNA templates were

Figure 4. (A) Poly(A) distribution was detected from 55 pmol RNA. (B) Poly(A) distribution detected from 250 pmol. The error bars represent the standard deviation from triplicate digests. More lengths are detected in higher RNA load (B), but the weighted average remains consistent across the two amounts. (C) Deconvoluted spectrum of a 250 pmol RNA poly(A) distribution highlighting the minimal salt adducts detected with no desalting prior to analysis.

Figure 5. Average weighted poly(A) tail length remains constant at 109 nt as the amount of digested RNA is increased. However, there are more species detected as the RNA load is increased.
characterized with a diagnostic restriction digestion method using an Agilent TapeStation (Figure 8). Sizing of DNA with the TapeStation is accomplished via the inclusion of low-molecular-weight and high-molecular-weight markers with each analytical sample. These data indicate that cloned poly(A) tails up to 150 bp are relatively stable, though the appearance of truncated species is increasingly apparent in templates with longer poly(A) tails. These templates were used in in vitro transcription

Figure 6. RNA (4100 nt with ∼100 nt poly(A) tail encoded) was spiked with a mixture of synthetic poly(A) standards containing lengths of 40, 60, and 80 nt. We can detect each spiked oligonucleotide as well as the average poly(A) tail distribution in our sample.

Figure 7. mRNA (1929 nt) was purchased from TriLink with an expected poly(A) tail of 120 nt. Triplicate 100 μg digests were prepared, and we calculated a weighted average of 123 nt for the sample. The error bars represent the standard deviation of the replicates.

Figure 8. DNA templates for GFP were created with varying poly(A) lengths (50, 80, 100, 120, and 150). Restriction enzymes were used to cleave and isolate the poly(A) containing fragment from the plasmid and run on an Agilent TapeStation. The sizing agrees well with the expected size. In larger poly(A) tail containing sequences, impurities of smaller sizes are observed.
reactions to generate GFP mRNA with different poly(A) lengths. We applied our mass spectrometry method to detect poly(A) tails in these mRNA samples. The results indicate that we are able to detect robust signals for each mRNA and the sizes detected are consistent with the templated length of poly(A) tails for the molecules. It has been previously reported that poly(A) 150 is unstable in the plasmid and a broad peak can be seen in the electropherogram (Figure 8).\textsuperscript{27} The RNA molecules synthesized from these templates were analyzed with the HRMS method and the poly(A) distributions for each sequence are shown in Figure 9. The weighted average poly(A) tail lengths were calculated for each sample and agree well with the measurements obtained with the DNA templates (Table 1).\textsuperscript{17}

### 4. CONCLUSIONS

Characterization of mRNA capping and tailing is critical in understanding the product quality and translation potential for mRNA therapeutics. The methods presented herein involving HPLC coupled with high-resolution mass spectrometry detection using the HFIP/TEA/TEAA mobile phase blend offer a faster alternative to previously reported methods for assessing these critical quality attributes. The shorter sample processing time and ability to analyze samples from a higher-throughput screening system (due to the lower amounts of the sample needed to run the assay) are equally beneficial. HPLC column performance has been acceptable with a typical column lifetime ranging between 1800 and 2000 injections before proactively being replaced. To date, we have run over 10,000 cap and tail samples and have not seen any performance issues with our mass spectrometer.

For capping analysis, we were able to detect a linear response for both capped and uncapped analytes when using the CleanCap analog in the molar equivalent ratio as reported by the vendor. The values were consistent with TriLink’s findings giving about 95% capping efficiency.\textsuperscript{28} The incorporation of an internal standard into the enzymatic digestion step alerts us when a sample has incomplete digestion and has helped to build more confidence in the assay.

For poly(A) tail analysis, we utilized a “chop-and-shoot” approach to simplify the sample preparation and dramatically substantially reduce the preparation time to detect the poly(A) tail mRNA sequence reliably with as little as 55 pmol. By eliminating oligo dT purification post-digestion, we obviate the bias introduced with the columns and were able to detect poly(A) lengths ranging from 50 to 150 nt, which we were unable to deconvolute when using the traditional HFIP mobile phases. Similar to Beverly et al.,\textsuperscript{20} we observed poly(A) distributions on our RNA sequences larger than those designed in the DNA template and can likely be attributed to transcriptional slippage by the RNA polymerase.\textsuperscript{29}

We recognize that a common challenge with the use of ion pairing agents in mass spectrometry is the persistence of contaminating ions in the mass spectrometer (102 in the case of TEA). Indeed, we see some of that with this assay. To overcome this, we routinely limit our scan range from 150 to 3000 m/z. On the occasions where we do need to scan a lower m/z range, we aggressively clean the system and/or utilize ion-exclusion lists to

### Table 1. Expected Poly(A) Length in GFP DNA Templates Compared to the Observed Weighted Average Poly(A) Length Using Our Method

| targeted Poly(A) length (nt) | weighted average Poly(A) length by mass spectrometry (nt) | expected size on tapestation (bp) | measured size on tapestation (bp) |
|-----------------------------|------------------------------------------------------------|----------------------------------|----------------------------------|
| 0                           | 0                                                          | 187                              | 192                              |
| 50                          | 55.2                                                       | 214                              | 218                              |
| 80                          | 85.2                                                       | 244                              | 244                              |
| 100                         | 104.3                                                      | 264                              | 258                              |
| 120                         | 120.7                                                      | 284                              | 278                              |
| 150                         | 140.2                                                      | 314                              | 301                              |

“The DNA fragments were cleaved with a restriction enzyme and the expected fragment size for the poly(A) containing species is listed as well as the calculated size by the TapeStation. The size of the restriction fragment without a poly(A) tail is expected to be 187 nt.
exclude this mass. We recommend that readers adopt similar strategies to overcome this obstacle.

**ASSOCIATED CONTENT**

* Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c00185.

This material includes the calibration curve of a properly $5'$-capped mRNA in the background of an uncapped mRNA of the same sequence; It also includes the Biopharma Finder deconvolution parameters used for the assays (PDF)

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**Notes**

The authors declare the following competing financial interest(s): Steven Strezsak, Alyssa Pimentel, Ian Hill, and Nicholas Skizim are all current or former employees of Greenlight Biosciences and hold equity positions in the company. Penny Beuning declares no competing financial interest.

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**REFERENCES**

(1) Wolff, J. A.; Malone, R.; Williams, P.; Chong, W.; Acsadi, G.; Jani, A.; Felgner, P. Direct Gene Transfer into Mouse Muscle in Vivo. *Science* 1990, 247, 1465–1468.

(2) Suschak, J. J.; Williams, J. A.; Schmaljohn, C. S. Advancements in DNA Vaccine Vectors, Non-Mechanical Delivery Methods, and Molecular Adjuvants to Increase Immunogenicity. *Hum. Vaccines Immunother.* 2017, 13, 2837–2848.

(3) Tandrup Schmidt, S.; Foged, C.; Smith Korsholm, K.; Rades, T.; Christensen, D. Liposome-Based Adjuvants for Subunit acines: Formulation Strategies for Subunit Antigens and Immunostimulators. *Pharmaceutics* 2016, 8, 7.

(4) Trepetoc, Z.; Geiger, J.; Plank, C.; Aneja, M. K.; Rudolph, C. Segmented Poly(A) Tails Significantly Reduce Recombination of Plasmid DNA without Affecting MRNA Translation Efficiency or Half-Life. *RNA* 2019, 25, 507–518.

(5) Faiman, B. Approved mRNA Vaccines for SARS-CoV-2. *J. Adv. Pract. Oncol.* 2021, 12, 17–18.

(6) Ramanathan, A.; Robb, G. B.; Chan, S.-H. MRNA Capping: Biological Functions and Applications. *Nucleic Acids Res.* 2016, 44, 7511–7526.

(7) Galloway, A.; Cowling, V. H. MRNA Cap Regulation in Mammalian Cell Function and Fate. *Biochim. Biophys. Acta, Gene Regul. Mech.* 2019, 1862, 270–279.

(8) Beverly, M.; Dell, A.; Parmar, P.; Houghton, L. Label-Free Analysis of MRNA Capping Efficiency Using RNase H Probes and LC-MS. *Anal. Bioanal. Chem.* 2016, 408, 5021–5030.

(9) Galloway, A.; Atthi, A.; Grzelka, R.; Darzynkiewicz, E.; Ferguson, M. A. J.; Cowling, V. H. CAP-MAP: Cap Analysis Protocol with Minimal Analyte Processing, a Rapid and Sensitive Approach to Analysing MRNA Cap Structures. 2019. https://doi.org/10.1101/2019.12.29.890368.48.

(10) Warminska, M.; Sikorski, P. J.; Kowalska, J.; Jemielity, J. Applications of Phosphate Modification and Labeling to Study mRNA Caps *Top. Curr. Chem.* 2017, 3751 https://doi.org/10.1007/s41061-017-0106-y.52.

(11) Muttach, F.; Muthmann, N.; Rentmeister, A. Synthetic MRNA Capping. *Beilstein J. Org. Chem.* 2017, 13, 2819–2832.

(12) Henderson, J. M.; Uijita, A.; Hill, E.; Yousif-Rosales, S.; Smith, C.; Ko, N.; McReynolds, T.; Cabral, C. R.; Escamilla-Powers, J. R.; Houston, M. E. Cap 1 Messenger RNA Synthesis with Co-Transcriptional CleanCap Analog by in Vitro Transcription. *Curr. Protoc.* 2021, I, No. e39.

(13) Grudzien, E.; STEPINSKI, J.; JANKOWSKA-ANYSZKA, M.; et al. Novel Cap Analogs for in Vitro Synthesis of MRNAs with High Translational Efficiency. RNA 2004, 10, 1479–1487.

(14) Cong, P.; Shuman, S. Mutational Analysis of MRNA Capping Enzyme Identifies Amino Acids Involved in GTP Binding. Enzyme Guanylate Formation, and GMP Transfer to RNA. *Mol. Cell. Biol.* 1995, 15, 6222–6231.

(15) Guo, P. X.; Moss, B. Interaction and Mutual Stabilization of the Two Subunits of Vaccinia Virus MRNA Capping Enzyme Coexpressed in Escherichia Coli. *Proc. Natl. Acad. Sci. U.S.A* 1990, 87, 4023–4027.

(16) Dreyfus, M.; Régnier, P. The Poly(A) Tail of MRNAs. *Cell* 2002, 111, 611–613.

(17) Wilusz, C. J.; Wormington, M.; Peltz, S. W. The Cap-To-Tail Guide to MRNA Turnover. *Nat. Rev. Mol. Cell Biol.* 2001, 2, 237–246.

(18) Riet, J.; Ramos, L. R. V.; Lewis, R. V.; Marins, L. F. Research Article Improving the PCR Protocol to Amplify a Repetitive DNA Sequence. *Genet. Mol. Res.* 2017, 16, No. gmr16039796.31.

(19) Hajnsdorf, E.; Kaberdin, V. R. RNA Polyadenylation and Its Consequences in Prokaryotes. *Philos. Trans. R. Soc., B* 2018, 373, No. 20180166.

(20) Beverly, M.; Hagen, C.; Slack, O. Poly A Tail Length Analysis of in Vitro Transcribed MRNA by LC-MS. *Anal. Bioanal. Chem.* 2018, 410, 1667–1677.

(21) Sutton, J. M.; Guimaraes, G. J.; Annavarapu, V.; van Dongen, W. D.; Bartlett, M. G. Current State of Oligonucleotide Characterization Using Liquid Chromatography—Mass Spectrometry: Insight into Critical Issues. *J. Am. Soc. Mass Spectrom.* 2020, 31, 1775–1782.

(22) Basiri, B.; van Hattum, H.; van Dongen, W. D.; Bartlett, M. G. The Role of Fluorinated Alcohols as Mobile Phase Critical Issues. *J. Am. Soc. Mass Spectrom.* 2017, 28, 190–199.

(23) Chen, B.; Mack, S. F.; Bartlett, M. G. The Effect of Organic Solvents on Desorption Efficiency for Oligonucleotides. *J. Am. Soc. Mass Spectrom.* 2013, 24, 257–264.

(24) Griffeycor, R. H.; Sasmor, H.; Greig, M. J. Oligonucleotide Charge States in Negative Ionization Electrospray-Mass Spectrometry
Are a Function of Solution Ammonium Ion Concentration. *J. Am. Soc. Mass Spectrom.* 1997, 8, 155–160.

(25) Chang, H.; Lim, J.; Ha, M.; Kim, V. Narry. TAIL-Seq: Genome-Wide Determination of Poly(A) Tail Length and 3’ End Modifications. *Mol. Cell* 2014, 53, 1044–1052.

(26) Liu, Y.; Nie, H.; Liu, H.; Lu, F. Poly(A) Inclusive RNA Isoform Sequencing (PAIso-seq) Reveals Wide-Spread Non-Adenosine Residues within RNA Poly(A) Tails. *Nat. Commun.* 2019, 10, No. 5292.

(27) Grier, A. E.; Burleigh, S.; Sahni, J.; Clough, C. A.; Cardot, V.; Choe, D. C.; Krutein, M. C.; Rawlings, D. J.; Jensen, M. C.; Scharenberg, A. M.; Jacoby, K. PEVL: A Linear Plasmid for Generating MRNA IVT Templates with Extended Encoded Poly(A) Sequences. *Mol. Ther.−Nucleic Acids* 2016, 5, No. e306.

(28) Laczkó, D.; Hogan, M.; Toulmin, S.; Hicks, P.; Lederer, K.; Gaudette, B.; Castaño, D.; Amanat, F.; Muramatsu, H.; Oguin, T.; Ojha, A.; Zhang, L.; Mu, Z.; Parks, R.; Manzoni, T.; Roper, B.; Strohmeier, S.; Tombácz, I.; Arwood, L.; Nachbagauer, R.; Karikó, K.; Greenhouse, J.; Pessaint, L.; Porto, M.; Putman-Taylor, T.; Strasbaugh, A.; Campbell, T.; Lin, P.; Tam, Y.; Sempowski, G.; Farzan, M.; Choe, H.; Saunders, K.; Haynes, B.; Andersen, H.; Eisenlohr, L.; Weissman, D.; Kramer, F.; Bates, P.; Allman, D.; Locci, M.; Pardi, N. A Single Immunization with Nucleoside-Modified mRNA Vaccines Elicits Strong Cellular and Humoral Immune Responses against SARS-CoV-2 in Mice. *Immunity* 2020, 53, 724–732.e7.

(29) Molodtsov, V.; Anikin, M.; McAllister, W. T. The Presence of an RNA:DNA Hybrid That Is Prone to Slippage Promotes Termination by T7 RNA Polymerase. *J. Mol. Biol.* 2014, 426, 3095–3107.