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sgRNA and Cas9 mRNA Characterization by Ion Pair-Reversed Phase Liquid Chromatography Coupled to HRMS

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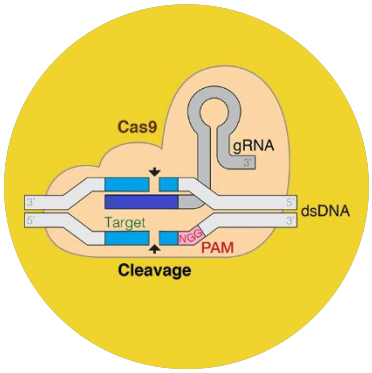


Introduction

RNA vaccines and CRISPR-based gene editing therapies are two scientific breakthroughs used to fight SARS CoV-2 and potentially treat many genetic diseases. The safety and efficacy of RNA vaccines and CRISPR-based gene editing products highly depend on the RNA stability and purity, thus making characterization a remaining challenge. Ion Pair-Reversed Phase (IP-RP) chromatography is the method of choice for oligonucleotide analysis, but conventional C18 columns are not often suitable for longer RNAs, such as single guide RNA (sgRNA) and messenger RNA (mRNA).

In this technical note, we present a workflow for the successful analysis of longer mRNAs using an sgRNA that targets the breast cancer gene 1 (BRCA1) gene and a CRISPR associated protein 9 (Cas9) mRNA as model systems. These RNAs were characterized by RNA mapping with RNase 4 enzymatic digestion and IP-RP coupled to high resolution mass spectrometry (HRMS) analysis using a Biozen™ Oligo column. Additionally, the sgRNA was analyzed in its intact form using a Biozen™ WidePore C4 column. These combined approaches provided: (1) sgRNA full sequence identification and chromatographic resolution of impurities and phosphorothioate (PS) diastereomers, and (2) Cas9-mRNA full sequence identification, including characterization of deletions and modifications at the 5'-UTR / 3'-UTR regions; the 5'-Cap identity confirmation, its partially capped species, and the Poly-A heterogeneity.

Analytes

1. **Hs.Cas9.BRCA1.1.AA**: *a 100nt long sgRNA containing the crRNA and tracrRNA sequences targeting BRCA1 gene and Cas9 Protein. mG*mA*mC*rGrUrCrUrGrUrCrUrArCrArUrUrGrArArUrGrUrUrUrUrArGrArGrCrUrArGrArArArUrArGrCrArArGrUrUrArArArArArUrArArGrGrCrUrArGrUrCrCrGrUrUrArUrCrArArCrUrUrGrArArArArArGrUrGrGrCrArCrCrGrArGrUrCrGrGrUrGrCmU*mU*mU*rU (*=Phosphorothioate bond, m= methyl group).*
2. **Cas9 mRNA**: *a 4129 nt RNA synthesized from de novo template/plasmid reduced dsRNA, containing enzymatically added Cap1 and 120 Poly-A tail.*

Sample Preparation

Optimized for 10 µg of RNA

1. In a 0.2 mL PCR tube, prepare a mixture of 10 µg RNA in 3 M urea to a final 10 µL volume.
2. In a thermal cycler, heat the RNA/urea mixture at 90 °C for 5 minutes followed by quick cooling to 25 °C.
3. In a 0.5 mL centrifuge tube dilute the denatured RNA mixture into 20 µL of 1.5 X NEB buffer™ r1.1, for a final 30 µL volume.
4. Add 1 µL of RNase 4 stock (50 U/µL)
5. Incubate the digest for 1 h at 37 °C.
6. Stop the RNase 4 digest with 1 µL of NEB RNase inhibitor, murine (NEB* #M0314) followed by incubation at room temperature for 10 minutes.
7. Dilute the final sample with water in 1:1 ratio and proceed to LC-MS for downstream analysis.

** NEB: New England Biolabs*

LC Conditions

	Digested sgRNA and mRNA	Intact sgRNA
Columns:	Biozen™ 2.6 µm Oligo C18 150 x 2.1 mm (00F-4790-AN)	Biozen™ 2.6 µm WidePore C4 150 x 2.1 mm (00F-4786-AN)
Mobile Phase:	A: 100 mM Hexafluoroisopropanol (HFIP) and 6 mM N,N-Diisopropylethylamine (DIPEA) in Water B: 7 mM Hexafluoroisopropanol (HFIP) and 0.5 mM N,N-Diisopropylethylamine (DIPEA) in Methanol / Water (80:20, v/v)	A: 100 mM Hexafluoroisopropanol (HFIP) and 6 mM N,N-Diisopropylethylamine (DIPEA) in Water B: 7 mM Hexafluoroisopropanol (HFIP) and 0.5 mM N,N-Diisopropylethylamine (DIPEA) in Acetonitrile / Water (70:30, v/v)

Gradients:

Time (min)	% B	Time (min)	% B
0	5	0	2
5	5	3	10
96	35	5	16
100	65	16	19
100.1	95	16.1	50
106	95	17	50
110	5	17.1	2
115	5	20	2

MS divert valve directs flow to waste for 5 minutes at start of run.

Flow Rate: 250 µL/min
Inj. Volume: 25 µL (Digested Samples)/ 1 µL (Intact gRNA)
Temp.: 75 °C
LC System: Thermo Scientific™ Vanquish™ UHPLC
Ionization: ESI Negative Mode
Detector: Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer

Data Analysis

Protein Metrics (Dotmatics) Digested Oligo Workflow was used for the analysis of enzymatically digested oligonucleotides using MS/MS acquisitions with high-resolution accurate mass.
Protein Metrics Intact Oligonucleotide Workflow was used for intact Poly-A analysis.

MS Instrument Method Settings

Resolution Full MS: 70,000
AGC Target Full MS: 3e6
MS Max IT, ms: 100
Scan Range, m/z: 580-1800
Loop Count: 15

MS2 Resolution: 17,500
MS2 AGC target: 1e5
MS2 Max IT, ms: 200

Isolation Window, m/z: 4
NCE: 10, 15 and 25
Intensity Threshold: 1e4
Peptide Match: Preferred
Dynamic Exclusion, s: 3
First Mass, m/z: 100
Charge Exclusion: 1, Unassigned
Exclude Isotopes: On
Loop Count: 8

Results and Discussion

Characterization of sgRNA: Intact sgRNA Analysis

Confirmation of the molecular weight of sgRNA was first carried out by intact analysis. For this purpose, 1 µL of a solution of 5 µM of sgRNA in water was injected onto a Biozen™ WidePore C4 400 Å HPLC column. This column was selected based on its benefits on recovery and separation of longer RNAs (see link). The biocompatible BioTi™ hardware mitigates adsorption, the wide pore core-shell particles provide fast mass transfer, and the pore size is ideal for longer oligonucleotides, such as sgRNA, which are typically ~100-120 nt in length. **Figure 1** shows the total ion chromatogram (TIC) and base peak chromatogram (BPC) obtained for the sgRNA sample, where a main peak was observed accompanied by an extensive impurity profile of shortmers and longmers. While some impurities were chromatographically resolved from the main peak at 16.5 min, the *m/z* data collected under the main peak shows the presence of various charged envelopes (**Figure 2**). After spectra deconvolution, peak assignment of the desired intact sgRNA (32,331 Da) was successful as well as n+1 and other impurities (**Figure 3** and **Table 1**).

Figure 1 . TIC (black) and BPC (purple) of the intact 100-mer sgRNA oligonucleotide with BRCA1 gene specificity analyzed on a Biozen™ Wide Pore C4 Column.

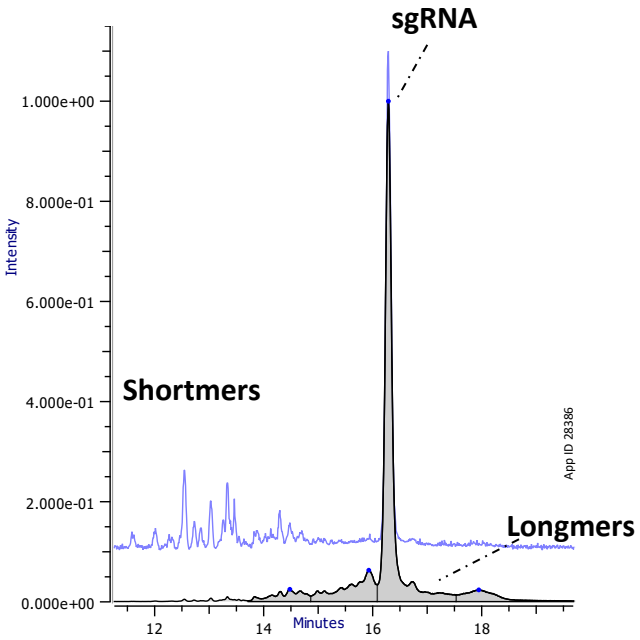


Figure 2 Charged envelope spectra collected under the main peak observed (16.3 minutes) with charge states ranging -47 at ~700 mz to -25 at ~1400 m/z.

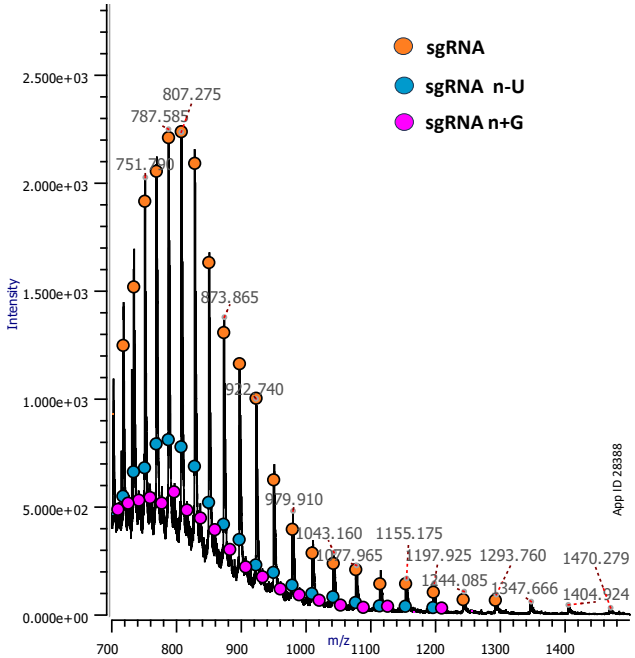


Figure 3 . Deconvoluted mass spectrum showing an experimental molecular weight of 32,331 Da for intact sgRNA.

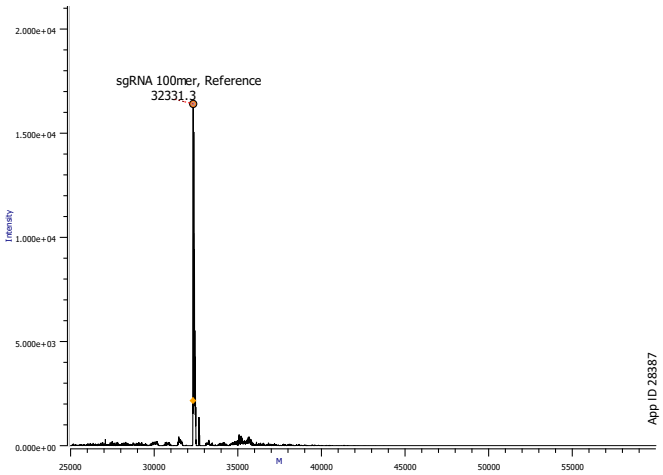


Table 1 . List of identified sgRNA and impurities with corresponding ion intensities, and mass errors obtained by HRMS intact mass analysis.

Oligonucleotide	Intensity	Monoisotopic Mass	ppm
sgRNA n-U	1.83E+03	32315.50	4.16
sgRNA	1.38E+04	32331.70	3.95
sgRNA n+ G	667	32676.75	3.81

The presence of impurities is not unexpected. sgRNAs are typically produced by solid phase synthesis with 5' and 3' end modifications to reduce degradation and improve translation efficiency. Phosphorothioate (PS) modifications are used in combination with 2'-O-methylations (2'OMe) for such purposes. It is a common occurrence that some PS modifications convert back to PO. Additionally, the PS modified oligonucleotides contain "2n-1" number of diastereomers due to the chiral center introduced by the phosphorothioate linkage, where "n" represents the number of phosphorothioate modifications in the oligonucleotide chain. Furthermore, solid-phase sgRNA synthesis is carried out in the 3' to 5' direction with sequence fidelity decaying towards the 5' terminus. This synthesis process produces truncations (n-x), additions (n+x) and other synthesis errors, with the number of impurities increasing with sgRNA length.

Characterization of sgRNA: sgRNA Mapping

After the initial assessment of sgRNA sample purity by intact mass analysis, further sample analysis is required to obtain sequence confirmation and in-depth impurity profiling. For this purpose, a Biozen™ C18 Oligo HPLC column was used to analyze sgRNA that was subjected to RNase 4 digestion. RNase 4 is a single-stranded endoribonuclease that cleaves RNA at Uridine-Purine (U/R) dinucleotide sites. The cleavage generates heterogeneous 3´ ends, producing a mixture of linear 3'-phosphate (3' lp) and/or cyclic 2',3'-phosphate (3' cp). Protein Metrics by Dotmatics Digested Oligonucleotide Workflow was used for data analysis. For identification purposes, correctly assigned charged envelope and reproducible RTs, with 10 ppm mass error cutoff was used, requiring at least 80 % MS2 fragmentation sequence coverage and unique oligonucleotide sequences to obtain oligonucleotide fragment identity. **Figure 4** shows the rich TIC of the digested sgRNA, demonstrating 100 % sequence coverage confirmation. The 5' and 3' ends of sgRNA each have 3 PS linkages, which will produce the presence of phosphorothioate diastereomers. Digestion products from the 5' and 3' ends would each then result in 2^{n-1} diastereomers, for a total of 4 peaks each. Our workflow was able to successfully resolve these peaks chromatographically as shown in the highlighted areas in **Figure 4**. Additionally, **Figure 5** shows the zoomed-in extracted ion chromatograms (EIC) of both 3' cp and 3' lp digestion products of *Gm*Am*Cm*GUCU,* including each of their four PS diastereomers that elute between 55 and 60 minutes. Being able to resolve cleavage fragments differing on their 3' end modification after cleavage and their diastereomeric arrangements showcases the excellent ability of the Biozen™ Oligo HPLC column to resolve even minute oligonucleotide differences and produce sharp intense peaks for MS analysis. This workflow was also able to confirm the presence of an addition of G at the 5' terminus and deletions(**Table 2**) as suggested by intact sgRNA analysis.

Figure 4 . Total Ion Chromatogram (TIC) of RNase 4 digested sgRNA on a Biozen™ Oligo HPLC column. Phosphorothioate diastereomers of 5' end *Gm*Am*Cm*GUCU* are highlighted in light blue and diastereomers of *3' end GCmU*mU*mU*U* are highlighted in light red.

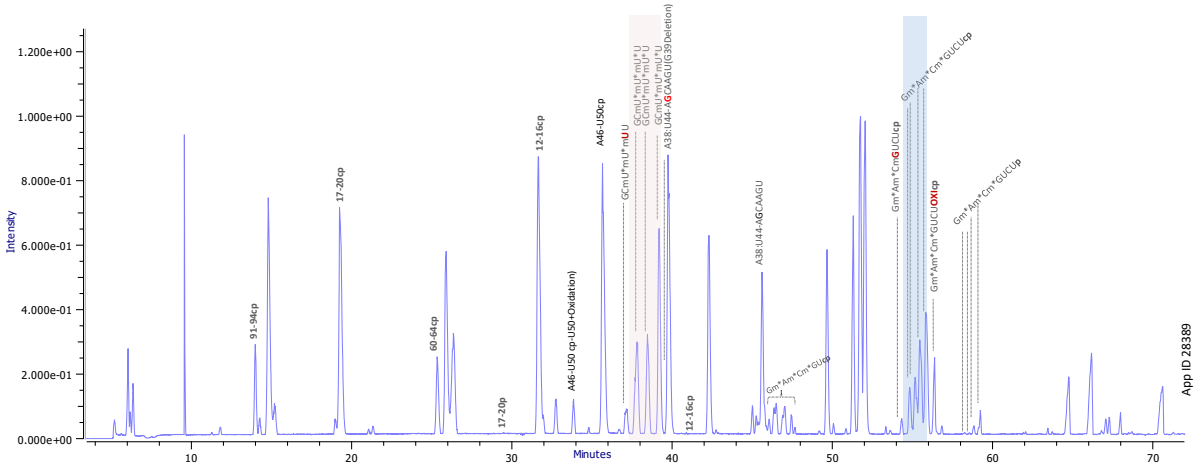


Figure 5 . EICs of 3'-linear phosphate (**blue**) and 2',3'-cyclic phosphate (**orange**) for cleavage fragment Gm*Am*Cm*GUCU.

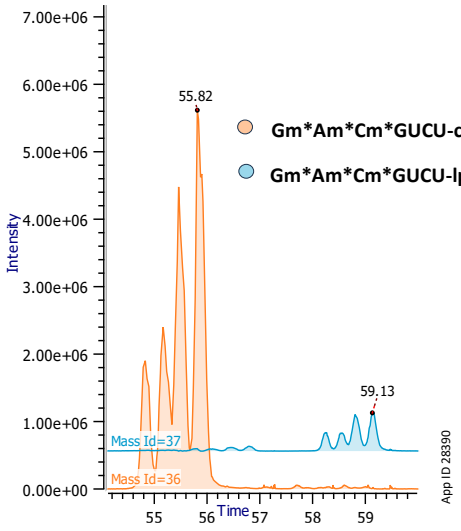


Figure 6 . Examples of other impurities: EICs of scrambled isomers (**left**) and PS diastereomers of 5' end oxidized at G4 (**Right**).

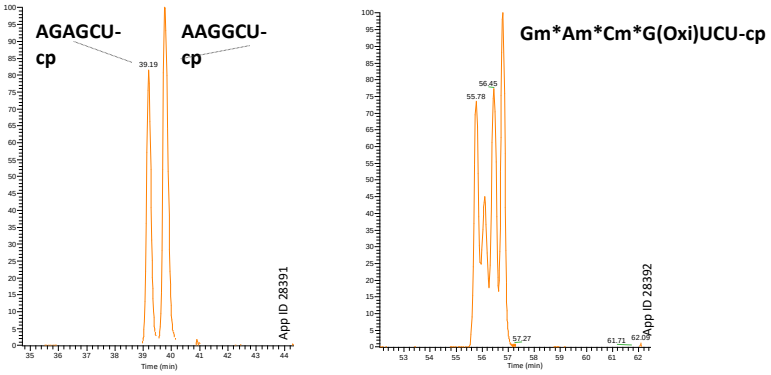


Figure 6 and **Table 2** show information on detected impurities. Interestingly, only Guanine residues were found to be oxidized. This can happen due to oxidative RNA damage when reactive hydroxyl radicals react with guanine in RNA, forming 8-hydroxyguanosine (8-OHG). Deletions at C60 and G39 which can hinder sgRNA activity were also found. Moreover, phosphorothioate conversion at U98 was also found. These PS to PO conversions (desulfurization) commonly occur during solid phase synthesis of oligonucleotides.

Table 2. Identified sgRNA modifications and chromatographically resolved isomeric species.

Modification	Seq Length	Site	RT	Monoisotopic Mass	Δ ppm
Phosphorothioate conversion	20	U98	74.12	6446.8408	-0.76
Deletion	14	C60	64.25	4201.5311	4.23
Oxidation (diastereomer 1)	7	G4	56.79	2347.2539	-3.74
Oxidation(diastereomer 2)	7	G4	56.45	2347.2539	-4.16
Oxidation (diastereomer 3)	7	G4	56.09	2347.2539	-3.64
Oxidation (diastereomer 4)	7	G4	55.76	2347.2539	-5.41
Deletion	10	C85	53.19	2954.4027	5.68
Isomer	6		39.78	1959.2665	1.74
Deletion	7	G39	39.73	1943.2716	1.46
Isomer	6		39.19	1959.2665	1.74
5' n+G	5	G1	59.42	2676.6600	3.73
Deletion	14	U90	52.90	4254.5880	1.95

Characterization of Cas9-mRNA

During mRNA production, there are at least three critical quality attributes (CQAs) that need to be fully characterized: 1.) confirmation of the complete nucleotide sequence, which is often thousands of nucleobases in length, as well as the assessment of the integrity of the added 2.) 5' cap and 3.) 3' poly-A tail. The 5' cap is relevant as it blocks or reduces exonuclease mediated degradation, initiating the translation process by which cells make therapeutic proteins and mitigate undesirable immune responses. The 3'-polyA tail is key for mRNA stability from exonucleases, facilitating the export from the nucleus to the cytoplasm and playing a role in translation efficiency. Here, the same workflow used for sgRNA sequence identification and impurity profiling was used for Cas9-mRNA and results are discussed below.

1. mRNA 5' Cap Integrity

The model Cas9-mRNA used here had a Cap-1 structure added by enzymatic addition using Vaccinia capping enzyme. Characterization of the 5' cap is challenging due to the heterogeneity among caps in any given sample, requiring particularly strong chromatographic resolution to distinguish and quantitate them. As a result, researchers often struggle to find optimal chromatography methods and columns capable of separating and characterizing 5' cap integrity. This technical note shows the identification of Cas9-mRNA Cap-1 structure and its variants (**Figures 7** and **8**, **Table 3**). Cas9-mRNA was enzymatically digested with RNase 4 which would theoretically produce *m^{7}GpppGmGGAU*, a 5 nt long oligonucleotide. However, endoribonuclease digestions are not perfect, and miss-cleavages are commonly present. So, the sequence *m^{7}GpppGmGGAUGGAUAAAAAAUAC* with 2 miss-cleavages was also considered during bioinformatic searches using Protein Metrics software.

Alternate workflows for 5'Cap identification by LC-MS consist of affinity enrichment using a DNA probe-directed analysis to capture the 5' cap species through the hybridization of the biotinylated DNA probe with the 5' cap mRNA. This is followed by the cleavage of the 5' cap and the isolation of hybridized RNA:DNA duplexes using streptavidin magnetic beads. In this work a different approach was used, aiming to find a one-method-fits-all approach in which the 5' cap , 5' and 3' UTRs, coding sequence and Poly-A tail are identified. In this study, Cap 1 and Cap 0 were both identified with a 3' lp and cp, with cp being the most abundant species.

Figure 7 . Extracted ion chromatograms of 5' cap species identified with 0 miss cleavages of the 3' cp species.

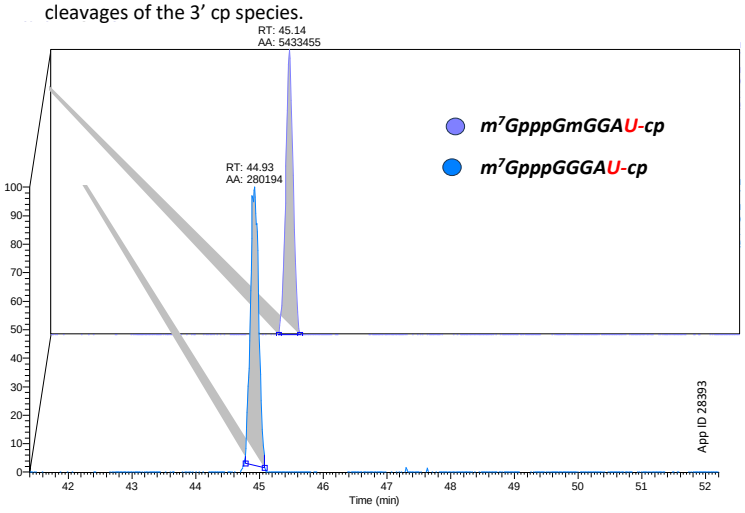
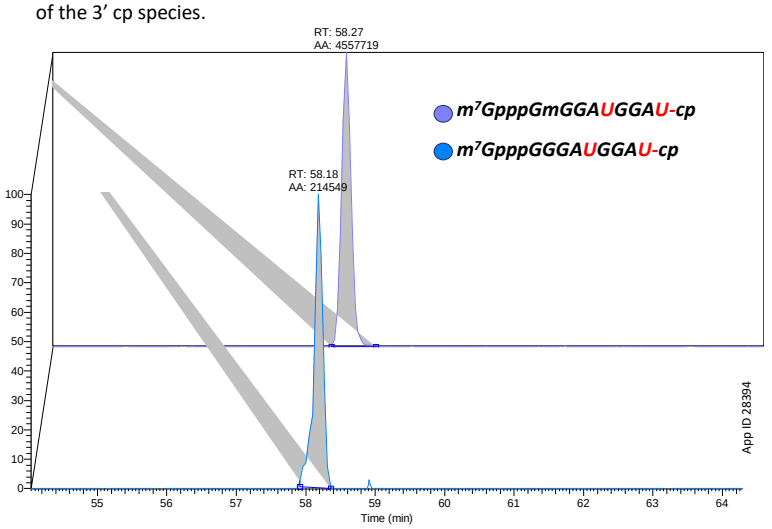


Figure 8 . Extracted ion chromatograms of 5' cap species identified with 1 miss cleavage of the 3' cp species.



Extracted ion chromatograms of both Cap 0 and Cap 1 are shown in **Figure 8.** Exact masses observed for the identified cap species with their respective error masses are listed in **Table 3**. While the di-phosphate, tri-phosphate and G Cap (GpppG) species were identified via accurate mass identification (≤ 5 ppm), these results were not considered confident identifications under our identification criteria, as their ion signals resulted in poor or absent MS2 fragmentation sequence coverage. As mentioned before, a 5' cap enrichment can be performed to obtain enriched signals and, hence, further 5' cap information. The presence of linear and cyclic phosphate species also reduces overall ion intensity. In this work, the cyclic phosphate species was found to be the most intense signal and was used for sequence identification. If a fully hydroxylated cap is desired, T4 Polynucleotide Kinase can be added to the digestion reaction mixture to obtain fully hydroxylated oligonucleotides fragments. Here, we show successful identification of chromatographically resolved Cap 1 and Cap 0 species in an hRNase 4 digested Cas9-mRNA sample. Relative abundance of each cap species was the same for each missed cleavage type, for example, Cap 1 with 1 missed cleavage and Cap 1 with 0 missed cleavages. Both resulted in ~95 % relative abundance irrespective of using 0 or 1 missed cleavage species for calculation.

Table 3 . 5' cap species identified and their corresponding ppm mass errors and relative abundance.

Cap Species	Oligo Sequence	Δ ppm	RT (min)	Monoisotopic Mass	Peak Area	Relative Abundance (Cap/ All Caps)	Missed Cleavages
Cap 0	m7GpppGGGAU-c.p.	3.32	44.93	2189.216	2.80E+05	4.90%	0
Cap 0	m7GpppGGGAUGGAU-c.p.	2.46	58.18	3514.389	2.15E+05	4.50%	1
Cap 1	m7GpppGmGGAU-c.p.	3.29	45.14	2203.232	5.43E+06	95.10%	0
Cap 1	m7GpppGmGGAUGGAU-c.p.	2.45	58.27	3528.404	4.56E+06	95.50%	1

2. mRNA Mapping

Following the same workflow as above, the entire Cas 9 mRNA sequence was studied. In silico digestion using up to 3 missed cleavages resulted in a total of 2175 possible oligonucleotide sequences. Experimental results that included cyclic and linear phosphate 3' ends as variable modifications resulted in a total of 3450 possible sequences and thus thousands of potential chromatographic peaks. **Figure 9** shows the complex base peak chromatogram and the sequence of well distributed and sharp intense peaks obtained for the digested Cas9 mRNA. Bioinformatic analysis was performed with Digested and Oligo workflows by Protein Metrics, as well as careful manual curation of the obtained experimental results. Peak identification was performed as accurately as possible via charged envelope and retention time data, using a +/- 10pmm mass error cutoff as a specification. Thus, requiring at least 80% MS2 fragmentation sequence coverage and unique oligonucleotide sequence to obtain oligonucleotide fragment identity. Using these conservative requirements, a Cas9-mRNA sequence coverage of 77 % was obtained. It is worth nothing that by only using accurate mass and not requiring the MS2 fragmentation sequence confirmation, the sequence coverage can be increased to 85 %. To obtain higher sequence identifications using our methodology, T4 Polynucleotide Kinase can be used in combination with hRNase 4 to obtain fully hydroxylated oligonucleotide sequences; thus, increasing signal intensity and increasing the odds of obtaining quality MS2 fragments which would directly translate to higher number of identifications. Aside from the 3' modifications due to cleavage, other mRNA impurities were also identified, such as deamination, oxidation and depyrimidination (**Table 4**). These results further support the observation that Biozen™ Oligo HPLC column provides excellent resolution of impurities, even when using moderately short gradients for separation of a complex ~4200 nt long mRNA digest.

Figure 9 . Base Peak Chromatogram (BPC) RNase 4 digested Cas9 mRNA separated on a Biozen Oligo HPLC column (A), Poly-A tail Total Ion Chromatogram (TIC) (B) and corresponding spectra (C).

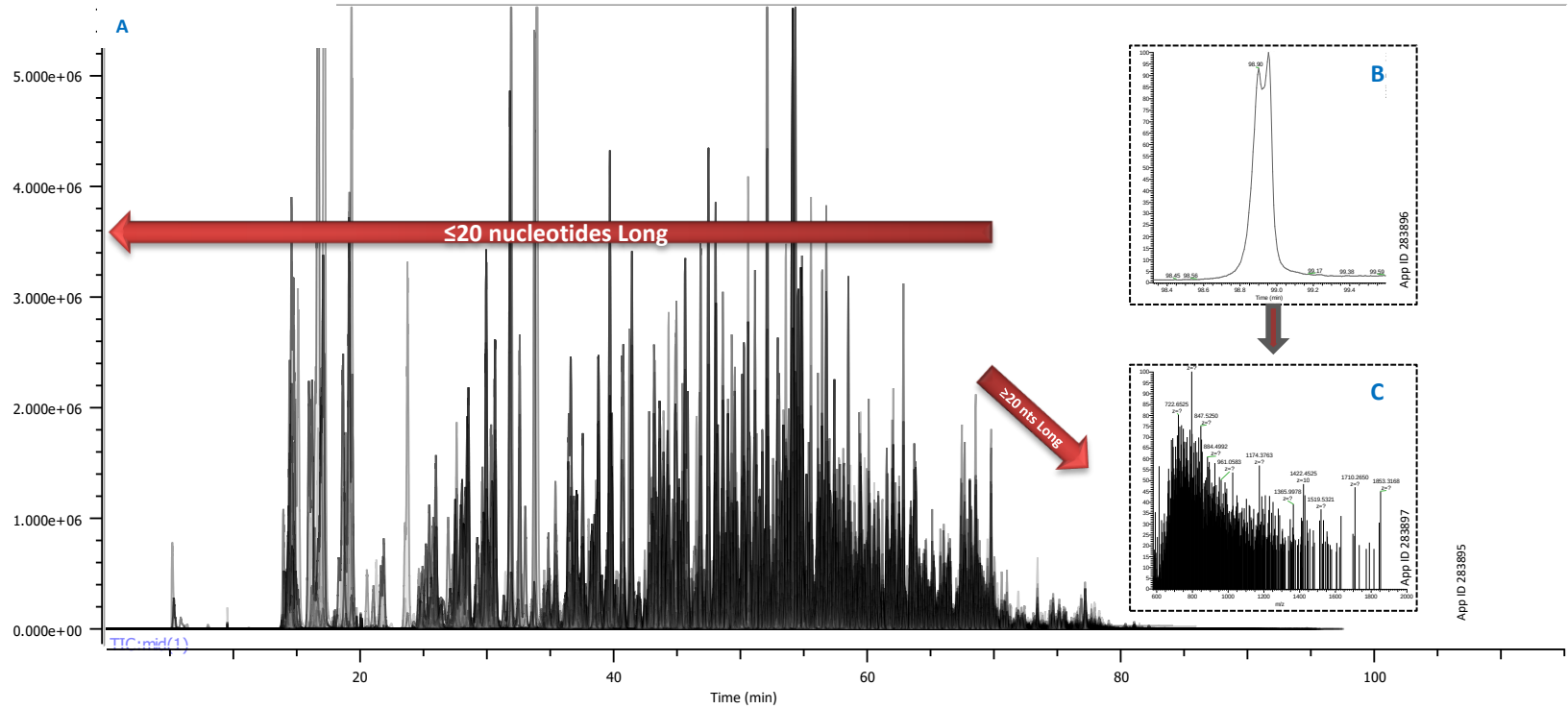


Table 4 . Selected identified cleavage fragments of Cas9 mRNA impurities and their modifications.

Stat #	End #	Oligo Sequence	Modification	Seq Length	Site	RT	Monoisotopic Mass	Δ ppm
2164	2176	GCAUGAACACAUU	Deamination	13	C2165	65.32	4169.541	4.86
2445	2464	AUUACCUGCAAAAUGGUCGU	Deamination	20	A2445	73.18	6411.806	6.14
3031	3041	GUAUGGCGAUU	Deamination	11	A3033	60.28	3568.421	4.49
3791	3805	AAACACUACCUGGAU	Deamination	15	A3791	68.23	4803.635	5.84
1934	1945	GACGAUAAAGUU	Depurination	12	A1935	62.54	3786.478	1.00
1089	1102	ACAUCGAUGGCGGU	Oxidation	14	A1089	66.51	4561.589	14.12
2459	2473	GGUCGUGACAUGUAU	Oxidation	15	G2459	68.68	4868.599	14.22
418	429	GCGUAAAAAACU	Acetylation	12	G418	65.31	3928.554	8.14
1517	1527	GAAAAAGUGCU	Acetylation	11	G1517	63.16	3639.507	6.57

3. Poly-A Tail Heterogeneity

The poly A tail of a mRNA is a repeat sequence of adenosine (A) that is grown at the 3' end of the 3' untranslated region. This tail provides additional stability to the "core" sequence, which will be translated into a functional protein, and plays an important role in ribosomal recruitment and translation efficiency. An optimal poly-A tail length of 100 – 150 nt is needed to ensure efficient protein expression. Given the inherent RNA stability challenges as well as current delivery challenges, the ability to understand the degradation of the poly A tail is essential. For this sample, the Poly-A tail was quite heterogenous, showing a broad split peak in the TIC (**Figure 10**). Under this broad peak, we observed a rich spectra of charged envelopes (**Figure 11**) that when deconvoluted, (**Figure 12**) show a high degree of heterogeneity, with hundreds of species belonging to additions and deletions of A, as accounted by each mass shift of ±329 Da. **Table 5** lists the species of Poly-A tail that were found with high mass accuracy. As stated earlier, alternate workflows to improve the analysis of the heterogenous Poly-A tails consist of its enrichment via poly(dT) magnetic beads. The WidePore C4 HPLC columns have also proven advantageous for analysis of long heterogenous Poly-A tails. Here, a "one-method-fits-all" workflow was used, and it was demonstrated that the major species of Poly-A tail can be characterized. Nonetheless, further clean-up is recommended if there is a need to further differentiate among the various Poly-A tail species.

Figure 10. Total ion chromatogram (TIC) of Poly-A tail at 99 minutes.

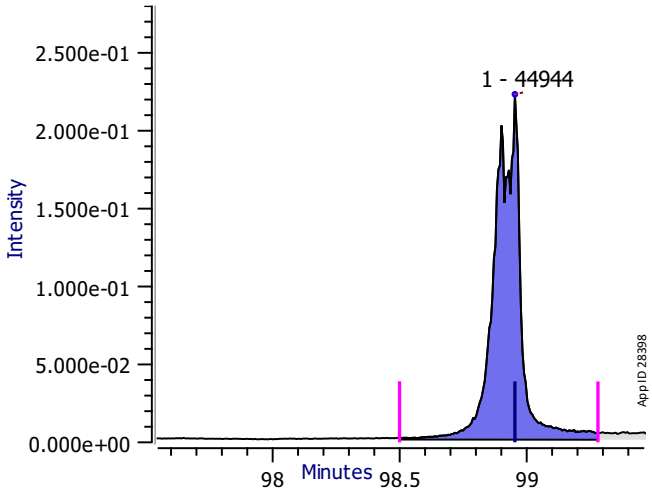


Figure 11. Charged envelope for identified Poly-A species.

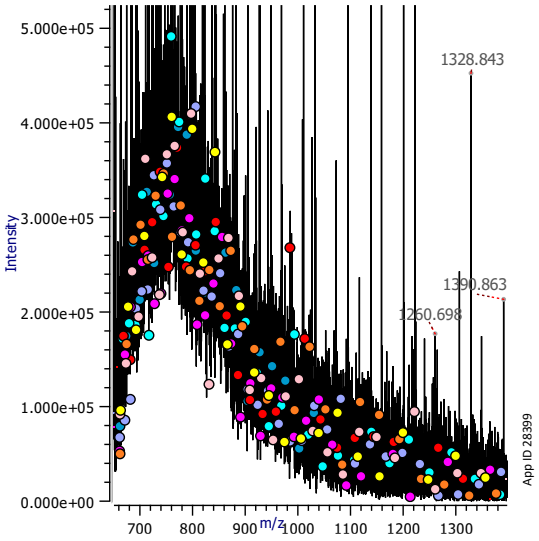


Figure 12. Deconvoluted spectra of charged envelops shown in **Figure 11**.

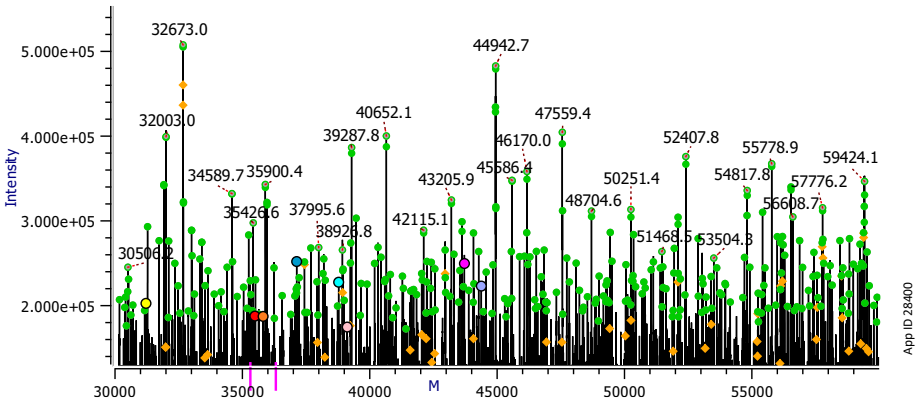


Table 5. List of PolyA tail species identified.

Oligonucleotide name	ppm Error	A Length	Monoisotopic Mass
PolyA 94	1.79	94	38764.98
PolyA 104	6.3	104	31198.56
PolyA 105	4.8	105	35475.56
PolyA 108	-1.52	108	35802.93
PolyA 112	-7.8	112	37119.14
PolyA 132	-5.9	132	43700.45
PolyA 134	1.81	134	44359.40

Conclusion

This technical note successfully demonstrates the ability of the Biozen™ Oligo C18 and Biozen™ Widepore C4 using a "one-method-fits-all" approach for the characterization of sgRNA and corresponding mRNA under ion-pair reversed phase conditions coupled with HRMS.

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