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Application News

Liquid Chromatograph Mass Spectrometer LCMS-9050

Analysis of mRNA 5′ Cap Structure Using a Quadrupole Time-of-Flight Mass Spectrometer

Junna Nakazono

User Benefits

- The LCMS-9050 quadrupole time-of-flight mass spectrometer and the LabSolutions Insight™ Biologics analysis software enable characterization of the 5′ cap structure of mRNA.
- The software enables simple confirmation of the fragment coverage of nucleic acids and their impurities.
- The software enables analysis of modifications and impurities that are set by the user.

■ Introduction

There has been increased attention on the new drug discovery modality of mRNA because of its efficacy in COVID-19 vaccines, and its applications for vaccines and other pharmaceuticals are expected to grow. Currently authorized mRNA vaccines are synthesized using *in vitro* transcription to add the Cap-1 structure (m7GpppRm-) on the 5′ end. This modification contributes to mRNA recognition, better efficiency of translation, and stability of mRNA in cell, making 5′ cap structure analysis an important element of mRNA quality controls.

This Application News presents a study of mRNA 5′ cap structure using the LCMS-9050 quadrupole time-of-flight mass spectrometer and LabSolutions Insight Biologics analysis software.

■ Samples

Given that mRNA is a large molecule, LC/MS analysis is typically done by analyzing fragments generated by cleavage enzyme reactions. In this study, the model sample consisted of Cap-1 structure mRNA with 36 bases (Cap-1 groups) obtained by *in vitro* transcription using plasmid DNA as a template. The 5′ cap modified unreacted RNA (pppR-) was also provided for analysis as an impurity.

■ Analytical Conditions

Analysis was performed using the Nexera™ XS inert and LCMS-9050 systems in Data Dependent Acquisition (DDA) mode. The analytical conditions are shown in Table 1.

Table 1 Analytical Conditions

UHPLC (Nexera XS inert)	
Column:	Shim-pack Scepter™ Claris C18-120* (150 mm × 2.1 mm I.D., 1.9 µm)
Mobile Phase A: Mobile Phase B:	95 mM HFIP, 5 mM DIPEA - water 70 mM HFIP, 5 mM DIPEA, 65% acetonitrile – water
Gradient Program:	B Conc. 5% (0-2 min) – 25% (22 min) – 90% (23-24 min) – 5% (24.1 -30 min)
Flowrate:	0.3 mL/min
Column Temp.:	60 °C
Injection Volume:	5 µL
MS (LCMS-9050)	
Ionization:	ESI negative
Mode:	MS *m/z* 550-2500, MS/MS (DDA) *m/z* 100-2500
Nebulizing Gas Flow:	3.0 L/min
Drying Gas Flow:	10.0 L/min
Heating Gas Flow:	10.0 L/min
Interface Temp.:	250 °C
DL Temp.:	250 °C
Block Heater Temp.:	400 °C

* P/N 227-31210-03

■ Setting the Analysis Parameters

LabSolutions Insight Biologics software is used to analyze nucleic acids and their impurities. First, the user creates a nucleic acid sequence in the parameter configuration window using the software presets for nucleobases, linkers, ribose, and modifications. Nucleobases, linkers, ribose, and base modifications can be added and removed in each tab as required. Once a sequence is entered, the software displays the molecular formula, monoisotopic mass, and structural formula (Fig. 1).

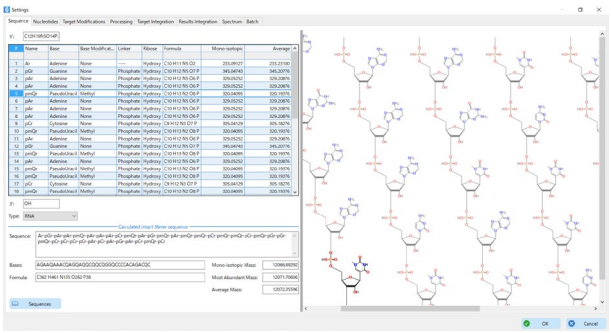


Fig. 1 Parameter Configuration Window

The Target Modifications tab is also used to select the anticipated impurities. In addition to impurities, such as different strand lengths, missing nucleobases, depurination/depyrimidination, deamination, and protecting groups, as well as adduct ions and unknown modifying groups, the software can also search for molecular changes added by the user. To enable detection of the 5′ cap modified unreacted group as the impurity in this study, "5′ uncapped" was added as the target modification (Fig. 2).

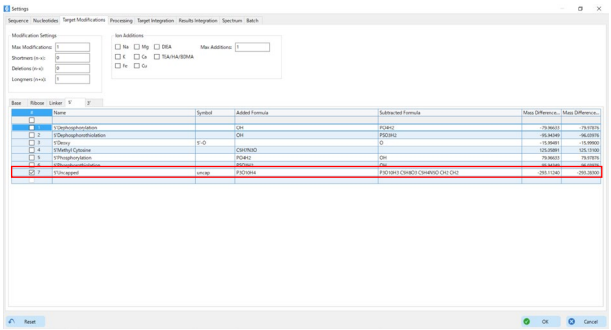


Fig. 2 Setting the Target Modification

■ Identifying Cap-1 and Unreacted Groups

Fig. 3 shows the component chromatogram of analyzed samples obtained by mixing Cap-1 group and the unreacted group by 0.5 µg each. The mass chromatogram is displayed as a component chromatogram, based on MS1 spectra and by combining signals from different valences and isotopes. Fig. 4 shows a result of multivalent ion analysis. Metal adducts were also detected. Moreover, longmers with an additional cytidine (C) (N+1 groups) were detected in the Cap-1 group and the unreacted group at ratios of 6.6 and 7.9%, respectively (Fig. 5). These groups were putatively identified as secondary reaction products generated by *in vitro* transcription.

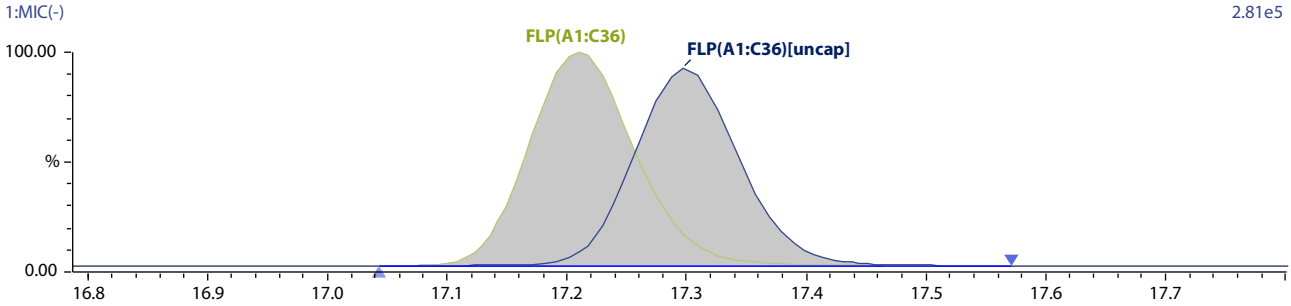
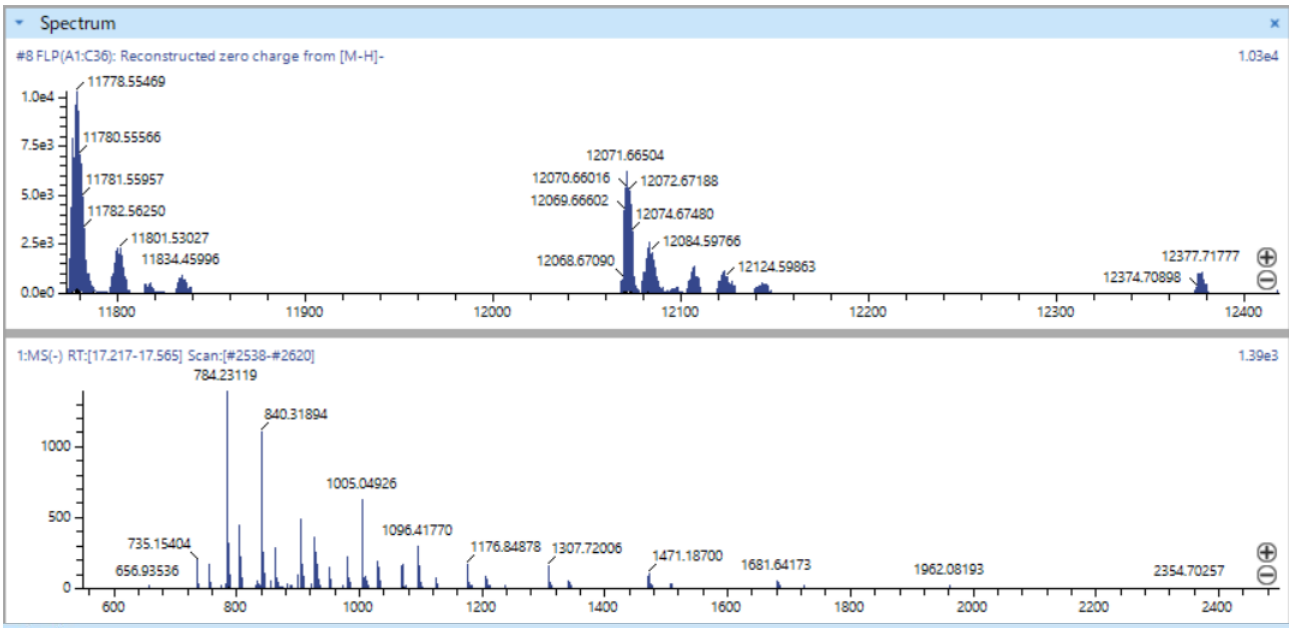
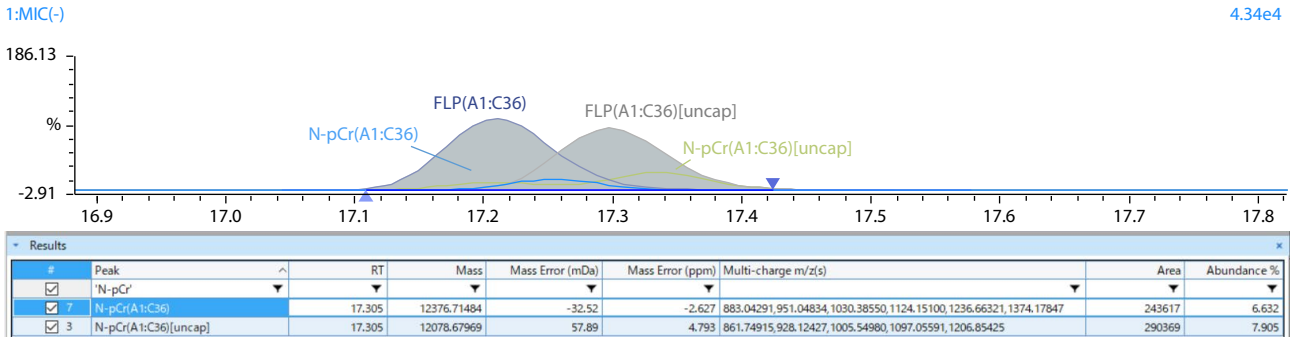


Fig. 3 Component Chromatograms of the Cap-1 Group and the Unreacted Group Mixture
FLP (A1:C36) indicates the Cap-1 group, and FLP (A1:36)[uncap] indicates the unreacted group.



#	Peak	RT	Mass	Mass Error (mDa)	Mass Error (ppm)	Multi-charge m/z(s)
1	FLP(A1:C36)	17.217	12071.66504	-41.02	-3.398	861.25305,927.58276,1004.96527,1096.41748,1206.15930,1340.28870
8	FLP(A1:C36)[-H+Fe]	17.305	12126.60840	-25.15	-2.074	865.17786,931.80902,1009.54456,1101.41016,1211.64636,1346.39905
7	FLP(A1:C36)[-H+K]	17.305	12109.59668	-64.91	-5.361	863.96436,930.50055,1008.12701,1099.86475,1209.95056,1344.49976
2	FLP(A1:C36)[-H+Na]	17.217	12093.65723	-30.78	-2.545	929.27576,1006.79657,1098.41431,1208.35901,1342.73938
3	FLP(A1:C36)[uncap]	17.305	11778.55469	-38.96	-3.308	840.31842,905.03589,980.53894,1069.77100,1176.84827,1307.71948
6	FLP(A1:C36)[uncap][-H+Fe]	17.305	11833.47852	-42.60	-3.600	844.24146,909.26062,985.11646,1074.76270,1182.34180,1313.82117
5	FLP(A1:C36)[uncap][-H+K]	17.305	11816.49316	-56.00	-4.739	843.02783,907.95374,983.70435,1073.21936,1180.64014,1311.93103
4	FLP(A1:C36)[uncap][-H+Na]	17.305	11800.51855	-57.04	-4.834	841.88806,906.72607,982.36859,1071.76709,1179.04443,1310.15991

Fig. 4 Results of Multivalent Ion Analysis
Top: Deconvoluted mass spectrum; Center: Mass spectrum; Bottom: Identification results



#	Peak	RT	Mass	Mass Error (mDa)	Mass Error (ppm)	Multi-charge m/z(s)	Area	Abundance %
7	N-pCr(A1:C36)	17.305	12376.71484	-32.52	-2.627	883.04291,951.04834,1030.38550,1124.15100,1236.66321,1374.17847	243617	6.632
3	N-pCr(A1:C36)[uncap]	17.305	12078.67969	57.89	4.793	861.74915,928.12427,1005.54980,1097.05591,1206.85425	290369	7.905

Fig. 5 Results of N+1 Group Identification in Cap-1 Group and Unreacted Groups Mixture
N-pCr (A1:C36) and N-pCr (A1:C36)[uncap] indicate the N+1 groups for the Cap-1 group and the unreacted group, respectively.

■ Sequence Identification

Fig. 6 shows the results of sequence coverage in the unreacted group, based on fragment ion information in MS/MS spectra. Upon identification of the selected target modification, the position of the modification is displayed in red. Identification of the N+1 group in the unreacted group also revealed characteristic fragment ions, including 3′ side C additions (Fig. 7). The detection of different strand lengths is indicated by the red inverse triangles.

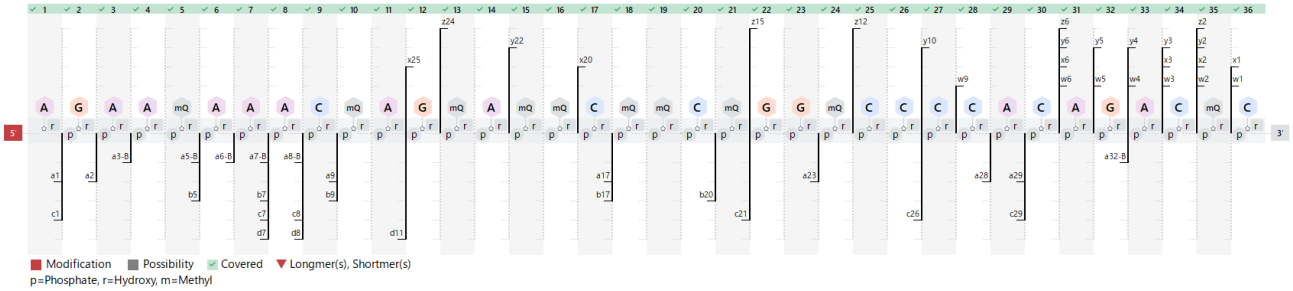


Fig. 6 Sequence Coverage of the Unreacted Group

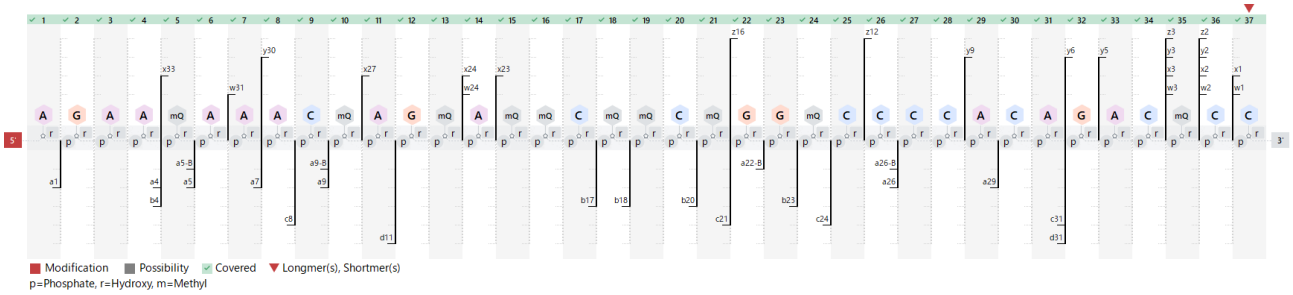


Fig. 7 Sequence Coverage of the N+1 Group in the Unreacted Group

■ Impurity Quantitation

Analysis of the samples, which were obtained by spiking the Cap-1 group with the unreacted group at concentrations of 0, 5, and 10%(w/w), was performed with the LabSolutions Insight Biologics software. The analysis showed the areas value had good linearity ($R^2 > 0.99$) (Fig. 8).

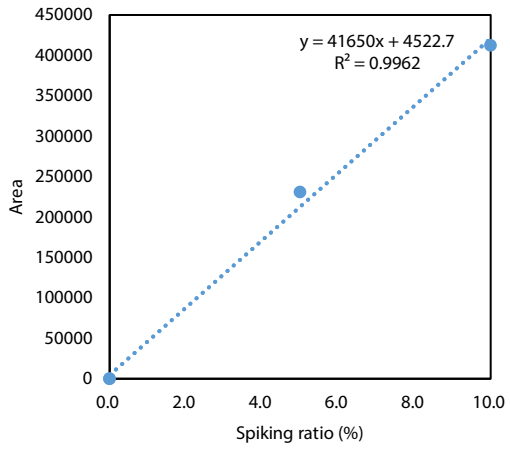


Fig. 8 Linearity of Unreacted Group (Impurity) Area Values

■ Conclusion

In this study, molecular weight identification and sequence analysis of 5′ cap modified mRNA was performed using the high-resolution LCMS-9050 mass spectrometer and LabSolutions Insight Biologics software. This instrument and software also enabled detection and sequence identification of impurities (N+1 group), in addition to detecting the Cap-1 and unreacted groups. LabSolutions Insight Biologics software simplifies the identification of modifications and deletions and additions with different strand lengths via the sequence coverage window.

The software also yielded area values of impurity with good linearity in the samples obtained by spiking the Cap-1 group with the unreacted group at concentrations of 0, 5, and 10%.

Related Applications

1. An Oligonucleotide Impurity Analysis Workflow Using LabSolutions Insight™ Biologics Software
   Application News No. 01-00595A-EN
2. Simple Analysis of Impurities in Oligonucleotide Therapeutics Using a Single Quadrupole Mass Spectrometer
   Application News No. 01-00656-EN

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