Poster Reprint

ASMS 2022
Poster number WP386

Comprehensive, Automated, and Integrated Software for Oligonucleotide Characterization and Sequence Confirmation

David Wong[1]; Peter Rye[2]; Stephen Madden[1]; Gordon Slysz[1] and Crystal Cody[1]

[1]Agilent Technologies, Inc., Santa Clara, CA
[2]Agilent Technologies, Inc., Lexington, MA

Introduction

Oligonucleotides including small interfering RNA, antisense oligonucleotides, aptamers, and CRISPR guides have become fast-growing modalities in recent years. Along with the development of these candidates has come the increased need for robust analytical methods and easy-to-use data analysis workflows to characterize them. Critical quality attributes of oligo samples include the determination of the target, confirmation of its sequence, and identification of impurities present – each of which can be time consuming, difficult, and tedious. As such, software that supports and automates these efforts can be of great value. In this work, we present novel, automated, and integrated software to support these workflows using HRAM MS data.

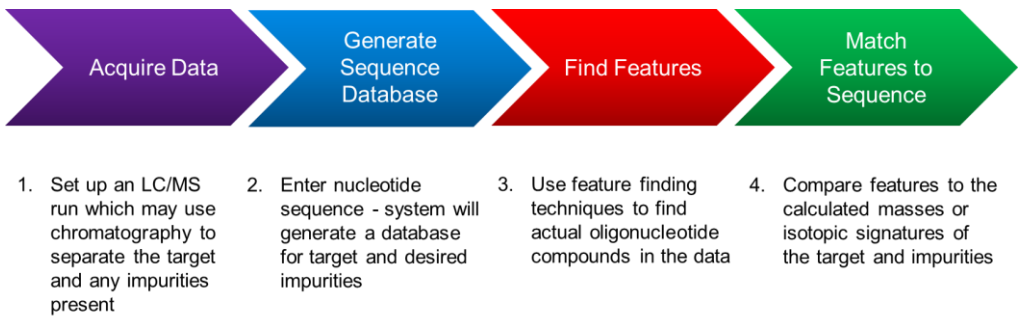

Figure 1. Target Plus Impurities (TPI) data analysis workflow in Agilent MassHunter BioConfirm 12.0 software

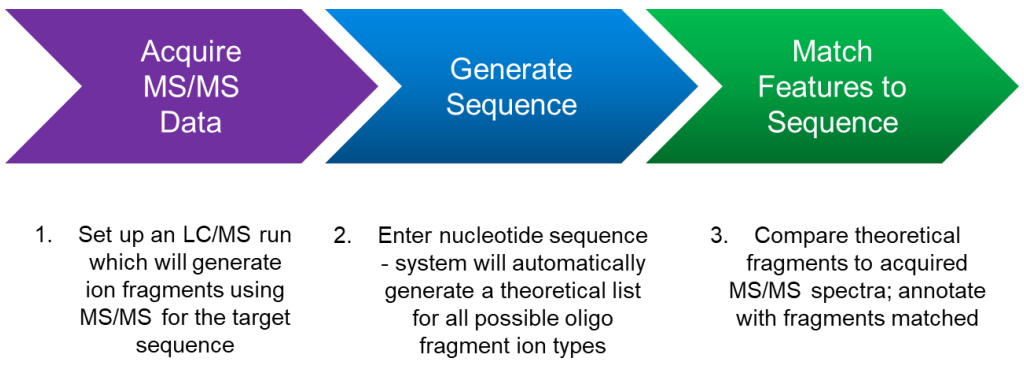

Figure 2. Oligonucleotide Sequence Confirmation data analysis workflow in Agilent MassHunter BioConfirm 12.0 software

Experimental

Oligonucleotide Samples

Oligonucleotide (DNA) Ladder Standard (part number 5190-9029), Oligonucleotide (RNA) Resolution Standard (part number 5190-9028), and RNA Standard (100-mer) were all obtained from Agilent.

21-mers and a 40-mer oligonucleotide were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) with standard desalting purification.

Most oligonucleotide samples were dissolved with DI water to 0.50 mg/mL without further purification.

Oligonucleotide Samples Analysis

LC/MS analyses of oligonucleotides were conducted on the Agilent 1290 Infinity II LC coupled to the 6545XT AdvanceBio LC/Q-TOF system. The AdvanceBio Oligonucleotides column was used with mobile phases containing traditional ion-pairing additives (triethylamine and hexafluoroisopropanol).

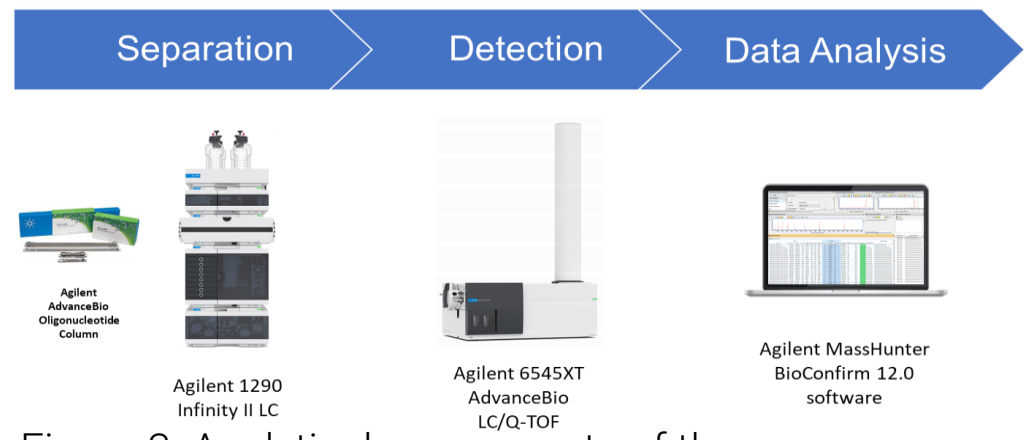

Figure 3. Analytical components of the oligonucleotide analysis - Target Plus Impurities (TPI) and Sequence Confirmation workflows.

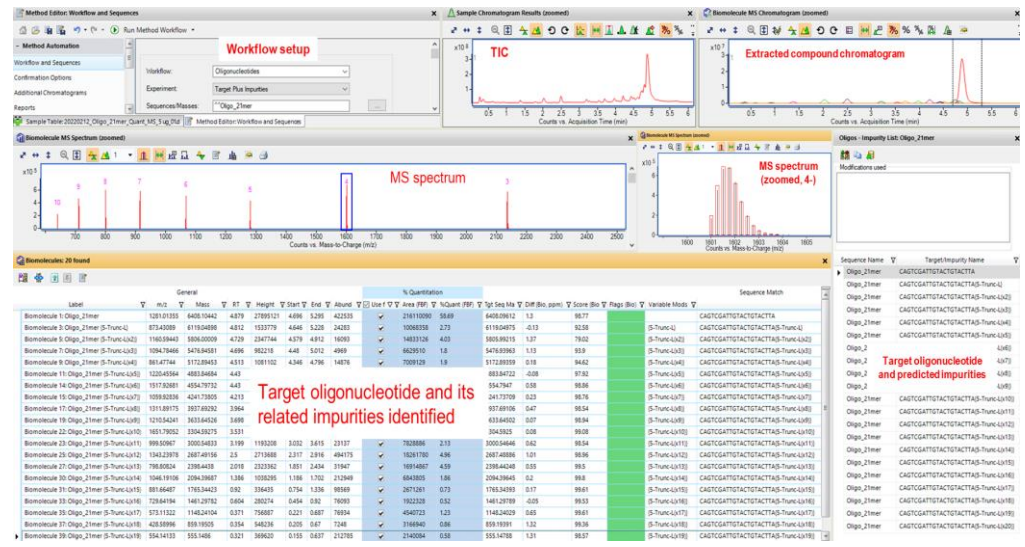

Figure 4. Overview of Agilent BioConfirm software, version 12.0 with Target Plus Impurities (TPI) workflow.

HPLC separation of various oligonucleotide standards

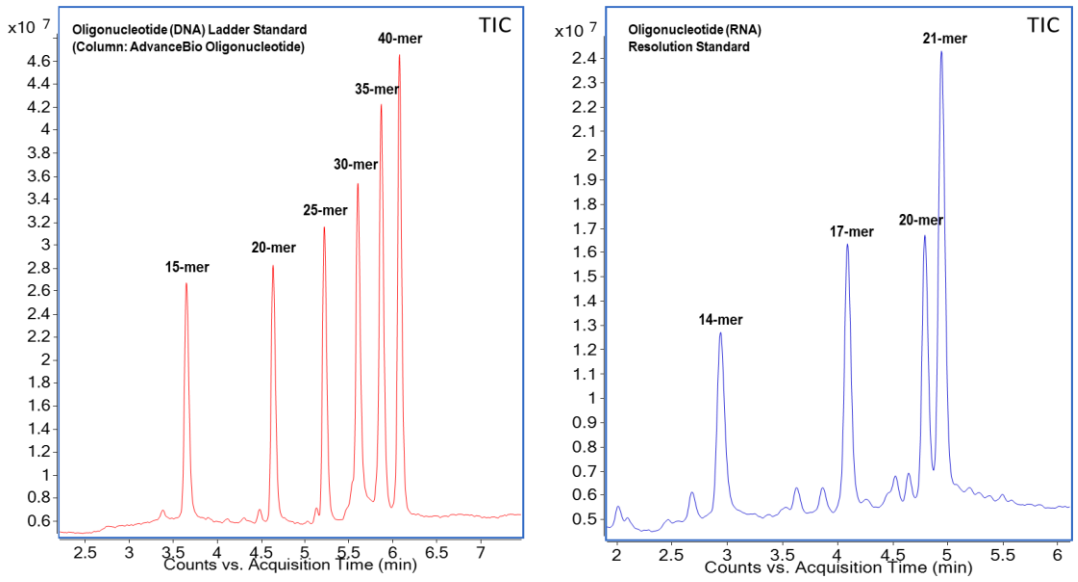

Figure 5. LC/MS Analysis of Agilent Oligonucleotide Ladder Standard (DNA) and Agilent Resolution Standard (RNA).

Oligonucleotide	Oligo Length	Sequence	Cal. Mass (Da)	Measured Mass (Da)	Mass Accuracy (ppm)
Oligonucleotide (DNA) Ladder Standard	15	TTTTT TTTTT TTTTT	4498.7348	4498.7319	-0.64
	20	TTTTT TTTTT TTTTT TTTTT	6018.9650	6018.9635	-0.25
	25	TTTTT TTTTT TTTTTTTTTT TTTTT	7539.1952	7539.1989	0.50
	30	TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT	9063.8431	9063.7988	-4.89
	35	TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT	10584.8111	10584.8065	-0.43
	40	TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT	12105.7790	12105.8295	4.17
Oligonucleotide (RNA) Resolution Standard	14	rCrArCrUrGrArArUrArCrCrArArU	4395.6479	4395.6429	-1.14
	17	rUrCrArCrArCrUrGrArArUrArCrCrArArU	5335.7670	5335.7623	-0.88
	20	rUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU	6275.8861	6275.8800	-0.97
	21	rGrUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU	6620.9335	6620.9263	-1.09
DNA-21	21	CAGTCGATTGTACTGTACTTA	6408.0961	6408.0952	-0.14
DNA-40	40	CCACGACCAAGTGACAGCAATGAATCGAGTCGAGATCCAT	12291.9558	12292.0000	3.60
RNA Standard (Long)	100	AACACCACCAUACAGUGCAGGUUUUAGAGCUAGAAAUAG CAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAA GUGGCACCGAGUCGGUGCUUUU	32178.5878	32178.9083	9.96

Table 1. List of oligonucleotides analyzed. Calculated masses highlighted in green are monoisotopic masses (matched using FBF) and the numbers highlighted in blue are average masses (matched using Maximum Entropy deconvolution).

LC/MS analysis of synthetic oligonucleotides

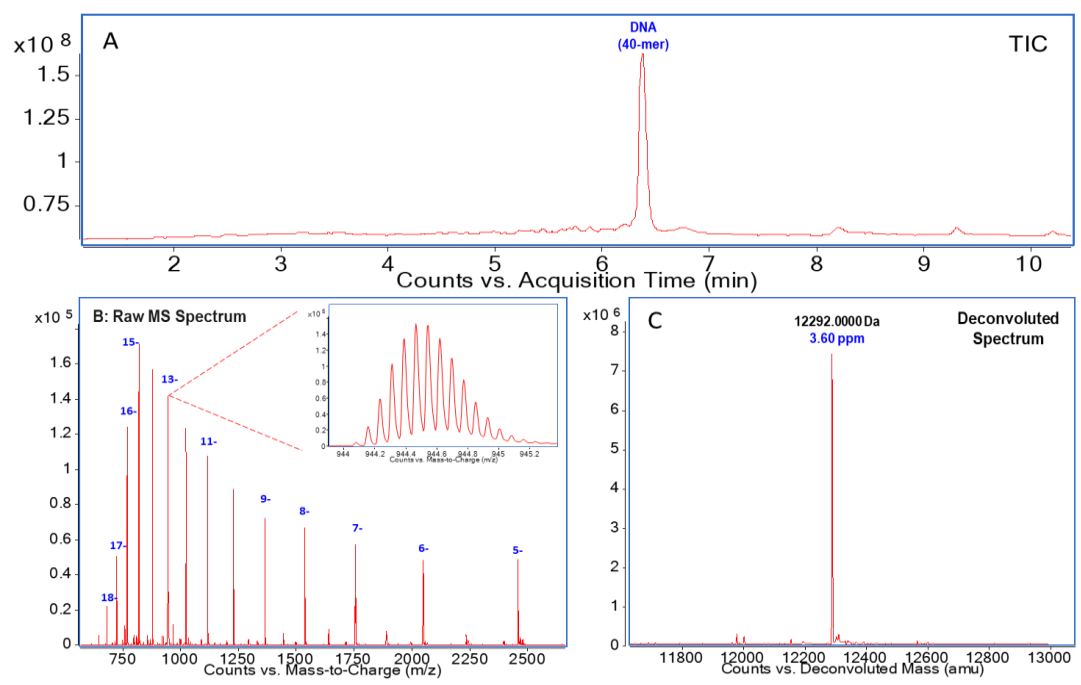

Figure 6. LC/MS analysis of synthetic oligonucleotide (DNA, 40-mer).

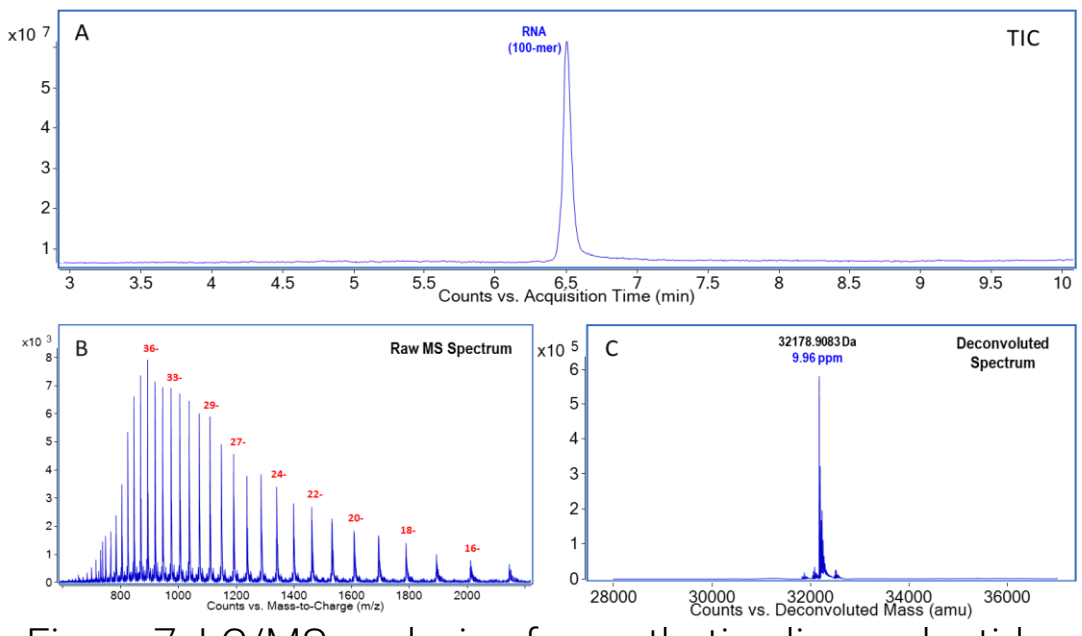

Figure 7. LC/MS analysis of a synthetic oligonucleotide (RNA, 100-mer).

LC/MSMS analysis and sequence confirmation of synthetic oligonucleotides

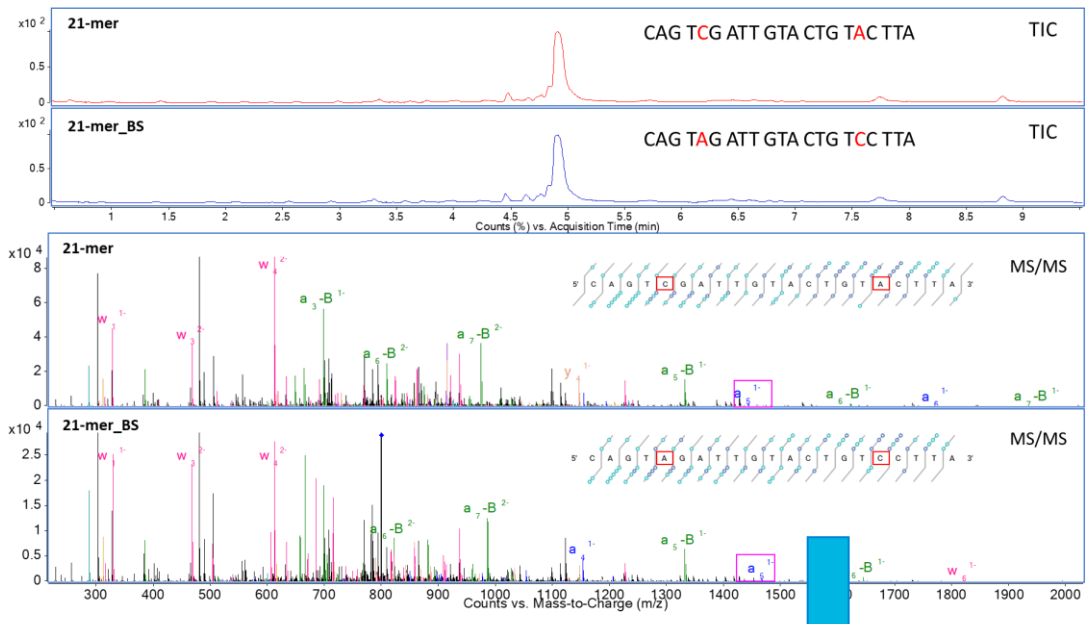

Figure 8. LC/MS/MS analysis of 21-mer and 21-mer_BS (Base Swap) oligonucleotides.

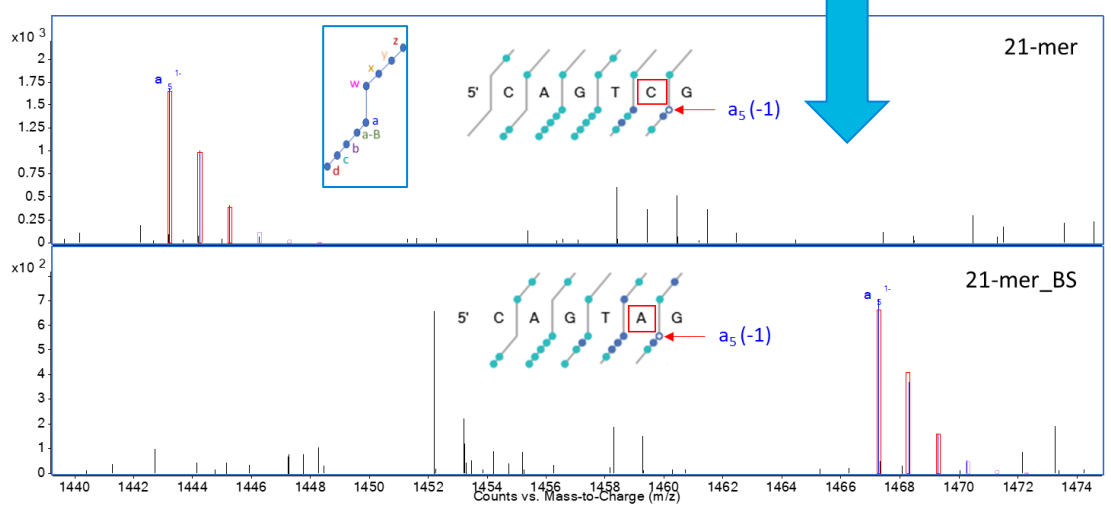

Figure 9. Sequence confirmation of 21-mer and 21-mer_BS (Base Swap) oligonucleotides (zoom-in spectrum).

Results and Discussion

LC/MSMS analysis and sequence confirmation of a heavily modified oligonucleotide

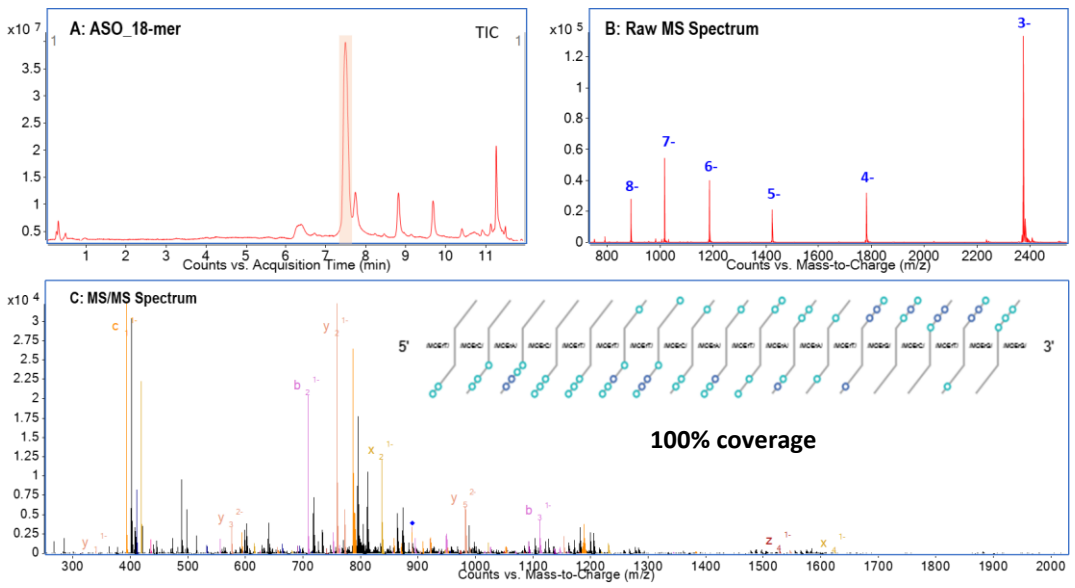

Figure 10. LC/MSMS analysis of a heavily modified oligonucleotide.

Oligonucleotides impurity analysis

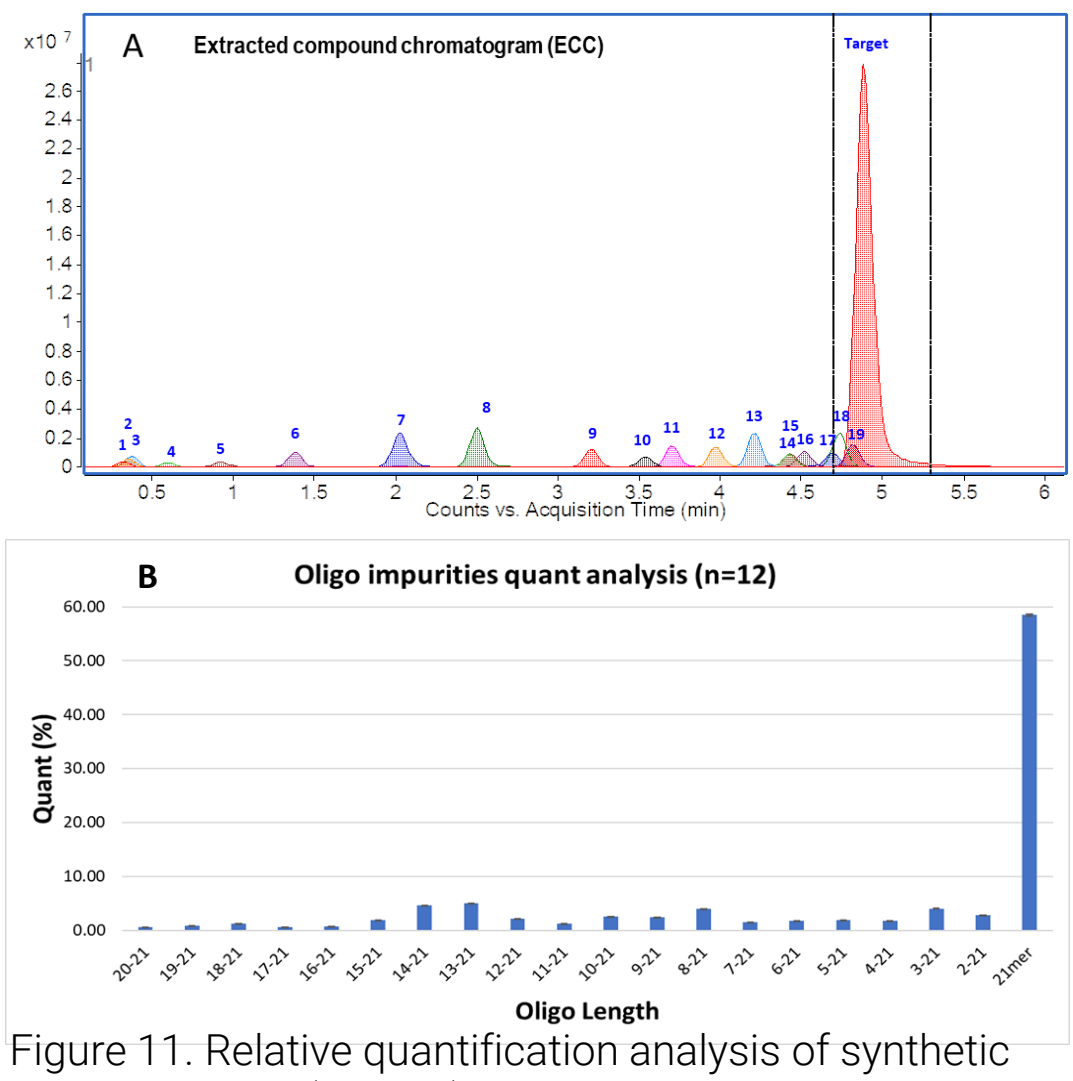

Figure 11. Relative quantification analysis of synthetic oligonucleotide (21-mer) and targeted impurities by the Find-by-Formula algorithm of Agilent BioConfirm software. (A) Extracted compound chromatography of the 21-mer oligonucleotide and its impurities. (B) Relative quantitation analysis results of the 21-mer oligonucleotide and its impurities.

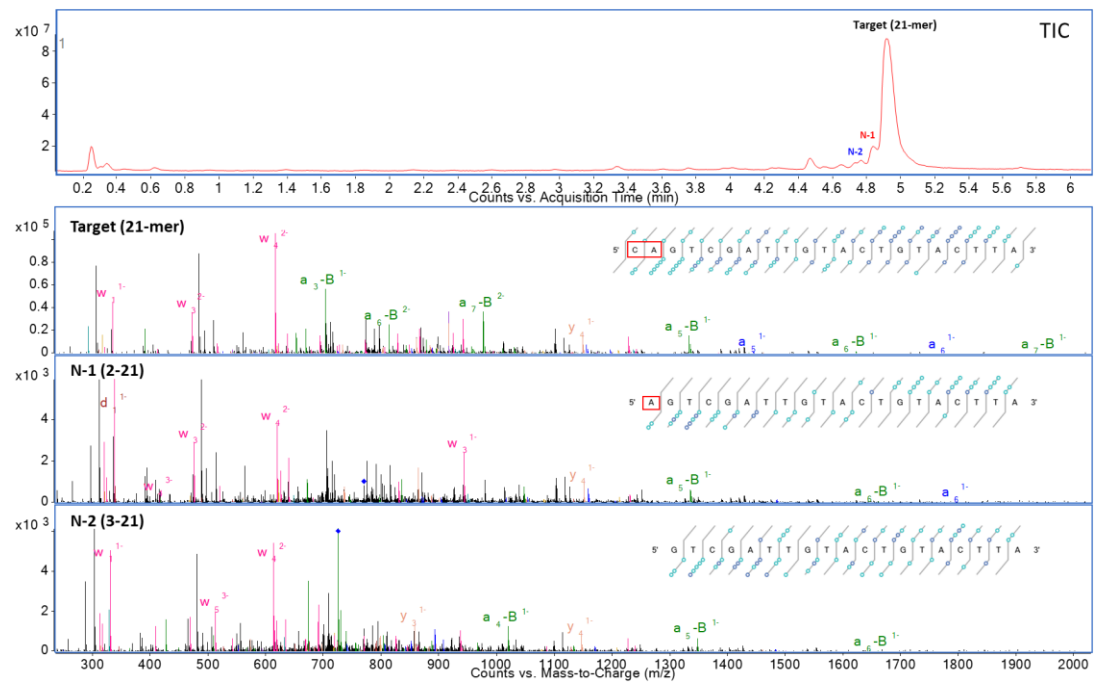

Figure 12. Sequence confirmation of 21-mer and its related impurities (N-1 and N-2).

Impurity Peak	Oligo Length	RT (min)	Calculated Mono Mass	Measured Mass	Avg Mass Accuracy (ppm)(n=12)	Avg %Quant (n=12)	Std Dev	RSD (%)	Sequence
1	20-21	0.321	555.1479	555.1486	1.21	0.57	0.01	2.39	TpA
2	19-21	0.354	859.1939	859.1950	1.09	0.89	0.02	1.76	TpTpA
3	18-21	0.371	1148.2403	1148.2410	0.81	1.28	0.02	1.44	CpTpTpA
4	17-21	0.604	1461.2979	1461.2978	0.15	0.53	0.01	1.18	ApCpTpTpA
5	16-21	0.920	1765.3439	1765.3442	0.57	0.72	0.01	1.72	TpApCpTpTpA
6	15-21	1.386	2094.3964	2094.3969	0.65	1.86	0.01	0.66	GpTpApCpTpTpA
7	14-21	2.018	2398.4425	2398.4438	0.69	4.61	0.04	0.91	TpGpTpApCpTpTpA
8	13-21	2.500	2687.4889	2687.4916	0.73	4.98	0.04	0.72	CpTpGpTpApCpTpTpA
9	12-21	3.199	3000.5465	3000.5483	0.33	2.14	0.02	0.75	ApCpTpGpTpApCpTpTpA
10	11-21	3.531	3304.5925	3304.5928	0.04	1.23	0.01	1.05	TpApCpTpGpTpApCpTpTpA
11	10-21	3.698	3633.6450	3633.6453	0.21	2.55	0.02	0.93	GpTpApCpTpGpTpApCpTpTpA
12	9-21	3.964	3937.6911	3937.6929	0.15	2.45	0.02	0.76	TpGpTpApCpTpGpTpApCpTpTpA
13	8-21	4.213	4241.7371	4241.7380	0.47	3.97	0.03	0.70	TpTpGpTpApCpTpGpTpApCpTpTpA
14	7-21	4.430	4554.7947	4554.7973	0.33	1.49	0.01	0.97	ApTpTpGpTpApCpTpGpTpApCpTpTpA
15	6-21	4.430	4883.8472	4883.8468	-0.23	1.74	0.01	0.85	GpApTpTpGpTpApCpTpGpTpApCpTpTpA
16	5-21	4.513	5172.8936	5172.8945	0.05	1.91	0.02	0.80	CpGpApTpTpGpTpApCpTpGpTpApCpTpTpA
17	4-21	4.696	5476.9396	5476.9458	1.22	1.81	0.01	0.79	TpCpGpApTpTpGpTpApCpTpGpTpApCpTpTpA
18	3-21	4.729	5805.9921	5806.0001	1.51	4.04	0.04	0.99	GpTpCpGpApTpTpGpTpApCpTpGpTpApCpTpTpA
19	2-21	4.812	6119.0498	6119.0490	-0.32	2.75	0.05	1.92	ApGpTpCpGpApTpTpGpTpApCpTpGpTpApCpTpTpA
Target	21mer	4.879	6408.0961	6408.1044	1.29	58.49	0.20	0.34	CpApGpTpCpGpApTpTpGpTpApCpTpGpTpApCpTpTpA

Table 2. Impurity analysis summary on 19 oligonucleotide impurities of a 21-mer synthetic oligonucleotide (n = 12).

Conclusions

- A highly automated and fully integrated oligonucleotide data (MS1 and MS/MS) analysis program was developed for comprehensive characterization of the targeted oligonucleotide and its related impurities.
- Our analytical results demonstrate that excellent chromatographic separation and mass accuracy (sub-ppm) for expected oligonucleotides were achieved.
- The LC/MS results also show accurate relative quantification of the observed oligonucleotides and their impurities, with very good reproducibility (RSD < 3%).
- We have achieved 100% sequence coverages on all oligonucleotides analyzed for sequence confirmation.

https://explore.agilent.com/asms
This information is subject to change without notice.

DE16130343

Published in USA, May 20, 2022

Agilent
Trusted Answers