

Mass Spectrometry

High-throughput analysis of oligonucleotides using a single quadrupole mass spectrometer for quality control

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Keywords

Single quadrupole mass spectrometer, ISQ EM, Vanquish Flex Binary UHPLC system, Chromeleon CDS, intact mass deconvolution, DNA, RNA, oligonucleotide analysis

Application benefits

This work provides a simple and comprehensive quality control workflow from synthesized oligonucleotide sample to a generated report for a specified target oligonucleotide mass.

Goal

Show step-by-step the analysis, deconvolution, and reporting of oligonucleotide synthesis quality control with a single quadrupole mass detector.

Introduction

Since the advent of Alexander Todd and his group's research of the oligonucleotide synthesis in the early 1950s¹ and the introduction of the solid phase phosphoramidite synthesis in the early 1980s,² public interest in the fields of immunology, virology, and RNA-based therapeutics utilizing custom-designed oligonucleotides has only increased. Subsequently, the commercial demand has amplified dramatically, especially with the emergence of the COVID-19 pandemic.³⁻⁴ To give perspective, single-stranded DNA has been a pioneering research tool for therapeutics for over 20 years, providing insight into precursor (pre)-mRNA splicing, gene expression, and immuno-pathways.⁵ As of 2020, there were more than 50 antisense oligonucleotide therapeutics in various development stages, 25 of which are in advanced stages (Phase II or III), and the United States Food and Drug Administration (US FDA) currently has approved 11.

Laboratories producing large arrays of customized DNA need to support this heightened throughput via increased automation and accuracy using intact mass determination for quality control. With this workflow from robotic DNA synthesis all the way through a confident pass/fail outcome for the expected sequence, Thermo Fisher Scientific offers a complete package consisting of the Thermo Scientific™ Vanquish™ Flex UHPLC system using the Thermo Scientific™ DNAPac™ RP column for

the separation. Determination of the intact oligonucleotide mass uses the Thermo Scientific™ ISQ™ EM Single Quadrupole Mass Spectrometer and is interpreted using the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) with the inclusion of the Intact Protein Deconvolution (IPD) engine and oligonucleotide analysis capabilities. Minor method optimizations provide cost savings and the reduction of 1,1,1-3,3,3-hexa-fluoro-iso-propanol (HFIP) and sodium adduct abundance.

Experimental

Chemicals

Chemical name	Supplier	Grade	Part number
1,1,1-3,3,3-hexa-fluoro-iso-propanol (HFIP)	Sigma-Aldrich	≤99% purity	105228
Methanol	Fisher Chemical	UHPLC-MS	A4581
Triethylamine (TEA)	Sigma-Aldrich	≤99% purity	T0886
Water	Fisher Chemical	UHPLC-MS	W81

Table 1. Oligomer sample array provided by GeneArt AG (part of Thermo Fisher Scientific), Regensburg, Germany.

All oligomers are 10 mM in water and were not desalted.

Oligo number	Sequence	Length (nt)	Theoretical average mass (Da)
1	AAGCCAGAGC	10	3206.0
2	CAATCTAAAGTATAT	15	4559.0
3	TCTCCCGGACGGAAAACCGCC	20	6047.9
4	AGGTAATTTGCGCTCATTGGGGGCC	25	7689.0
5	CCGGCCTATGGCCCACAATGTAAAGAATTA	30	9184.0
6	GCCCGTGGTAAAGCAGTTCACGTGTACATAGTTGT	35	10802.0
7	GCCCATAATTGAGCCCCGCTGCCGACGAGCGGCTTTGTGC	40	12249.9
8	CCCTGAATTAAGGGGGCAGCCCCTTAATGAATGCCCGGACTCGAA	45	13839.9
9	TAAACTGTTTATCGGGGCTCAAATCTTAGGCCTAGGCAGGATCCCGTAAG	50	15425.0
10	ATAATCGAGAATTGGTATCGATTCCGGGGCCACCCACAAGTCCGGTACACCAACCG	55	16897.9
11	CACACCTCGAAGAGTATTCCGTCCCGGAGCTGGTTAGGTGACTAACACTGCAAATTCTCT	60	18394.9
12	GGGGCGCTCTATCTTCCATC	20	6059.9
13	CCCGAGCGGAGTTTTGCGATAGTACACCAACCGAGCATCTCGAATTAAGGCCTG	55	16928.9

Sample handling

Item name	Part number
Thermo Scientific™ 11 mm plastic crimp/snap top autosampler vials	C4011-13
Cap with septum (Silicone/PTFE), Fisher Scientific™	13-622-292
Thermo Scientific™ 96-well plate	10547781

Instrumentation

Module	Part number
Vanquish Flex UHPLC system consisting of:	
System Base Vanquish Horizon/Flex	VH-S01-A-02
Vanquish Binary Pump F 35µL Mixer kit	VF-P10-A-01 6044.3870
Vanquish Split Sampler FT	VF-A10-A-02
Vanquish Column Compartment H Active Pre-Column Heater	VH-C10-A-02 6732.0110
Vanquish Variable Wavelength Detector F 2.5 µL SST flow cell	VF-D40-A 6077.0360
2-Position/6-Port Switching Valve (1500 bar)	6036.2520
ISQ EM single quadrupole mass detector	ISQEM-ESI

Sample preparation

The samples were provided in a 96-well plate. They were collected directly from the DNA synthesizer and were injected neat.

Mobile phase preparation

Glassware was cleaned by standard laboratory means with the addition of solvent rinses by adding water, shaking for 1 minute, and emptying; then, adding methanol, shaking for 1 minute, and emptying; and finally adding water, shaking for 1 minute, and emptying.

- Preparation of Eluent A

Eluent A was prepared by mixing neat HFIP (variable concentration) and a 1,000 µL portion of neat triethylammonium acetate (TEA) to water make a 1,000 mL volume. The eluent was freshly prepared every three days.

- Preparation of Eluent B

Eluent B was prepared by mixing neat HFIP (variable concentration) and a 1,000 µL portion of neat triethylammonium acetate (TEA) to methanol make a 1,000 mL volume. The eluent was freshly prepared every three days.

Chromatographic conditions

Table 2. Chromatographic conditions

Parameter	Value		
Column	DNAPac RP 2.1 × 50 mm, 4 µm (P/N 088924)		
Mobile phase	A: HFIP (0.01, 0.1, 0.5, 1.0, 2.0%), 0.1% TEA, in water B: HFIP (0.01, 0.1, 0.5, 1.0, 2.0%), 0.1% TEA, in MeOH		
Gradient	Time (min)	A	B
	0	99	1
	0.4	99	1
	0.4	75	25
	1.0	75	25
	1.0	0	100
	1.6	0	100
	1.6	99	1
	4	99	1
Flow rate	0.7 mL/min		
Column temperature	70 °C, forced air mode 70 °C, active pre-heater		
Autosampler temperature	4 °C		
Autosampler wash solvent	10% MeOH in water		
Injection volume	2 µL		
UV detector settings	λ=260 nm, 100 Hz		

MS settings

Table 3. Instrument and scan settings for the mass spectrometer used for the final sample analysis

Parameter	Value
HESI source settings	
Vaporizer temperature	350 °C
Ion transfer tube temperature	350 °C
Source voltage	-3,000 V
Sheath gas pressure	75 psig
Aux gas pressure	7.5 psig
Sweep gas pressure	0 psig
Scan settings	
Mass range	600–2000 <i>m/z</i>
Dwell/Scan time	0.5 s
Polarity	Negative
Spectrum type	Profile
Source CID voltage	0 V

The vaporizer temperature, transfer tube temperature, sheath gas/auxiliary gas pressures, and spray voltage were optimized by maximizing the peak area associated with the most abundant charge state. The instrument source settings were optimized at the beginning of experiments using Custom Injection Variables in Chromeleon CDS. This order of optimization is represented in

Table 4. It is important to note that the auxiliary gas pressure was always 10% that of the sheath gas pressure. Subsequently, the HFIP concentration was modified to improve the quality of the spectra. Finally, the source settings were optimized again at the new HFIP concentration.

Table 4. Variable source parameters in MS setting tuning

Order	Source parameter	Optimization range	Increments
1	Vaporizer temperature	300 to 450 °C	50 °C
2	Transfer tube temperature	300 to 400 °C	50 °C
3	Sheath gas (auxiliary gas)	50 to 80 psig (5 to 8 psig; 10% of sheath gas)	5 psig (0.5 psig)
4	Spray voltage	-1,000 to -5,000 V	1,000 V

#	UV_VIS_1	Name	Position	Volume [μL]	*VaporizerTemp [°C]	*TransferTubeTemp [°C]	*SheathGas [psig]	*SprayVoltage [V]	Instrument Method
1		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	300	300	75	-3000	HFIP Method v16 - ISQ Scouting
2		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	350	300	75	-3000	HFIP Method v16 - ISQ Scouting
3		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	400	300	75	-3000	HFIP Method v16 - ISQ Scouting
4		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	450	300	75	-3000	HFIP Method v16 - ISQ Scouting
5		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	300	350	75	-3000	HFIP Method v16 - ISQ Scouting
6		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	350	350	75	-3000	HFIP Method v16 - ISQ Scouting
7		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	400	350	75	-3000	HFIP Method v16 - ISQ Scouting
8		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	450	350	75	-3000	HFIP Method v16 - ISQ Scouting
9		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	300	400	75	-3000	HFIP Method v16 - ISQ Scouting
10		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	350	400	75	-3000	HFIP Method v16 - ISQ Scouting
11		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	400	400	75	-3000	HFIP Method v16 - ISQ Scouting
12		Sample G10 - 260nm with ISQ - 2μL injection - 55mer	G:F2	2.00	450	400	75	-3000	HFIP Method v16 - ISQ Scouting

Figure 1. Using custom variables for method optimization. In Chromeleon CDS, custom injection variables can be defined. Users can find Custom Injection Variable tutorials as the top search results for “create custom variables” and “use custom variables” in Chromeleon Help. Subsequently, selected method parameters are not set in the instrument method but in the sequence table. This allows for faster method optimization without the need to create multiple instrument methods. In this experiment, multiple Custom Injection Variables were used for iterative optimization of source parameter settings, according to Table 4. Custom Injection Variables are denoted by an asterisk before the parameter name in the sequence table. Inserted variables are as follows: VaporizerTemp (orange), TransferTubeTemp (blue), SheathGas (purple), SprayVoltage (yellow). A previously released technical note presents the same ISQ EM method settings approach applied to a different HFIP concentration and a 55-mer oligonucleotide.⁶

Chromatography Data System

Chromeleon CDS was used for data acquisition and analysis. The ISQ EM mass spectrometer is fully integrated into Chromeleon software, which was used for system operation, subsequent data analysis, and deconvolution using the integrated Intact Protein Deconvolution feature. This feature is also intended for oligonucleotides specifically with the negative charge and peak model setting (Table 5).

The obtained MS chromatograms were analyzed with the IPD settings shown in Table 5.

Table 5. Intact Protein Deconvolution settings

Parameter	Value
Peak retention window	0.7–0.8 min
Algorithm	ReSpect™
Output mass range	2,000–20,000 Da
Deconvoluted spectra display mode	Isotopic Profile
Model mass range	2,000–20,000 Da
Deconvoluted mass tolerance	100 ppm
Peak model	Nucleotide
Resolution	Raw File Specific
Charge carrier	H ⁺
Charge high	30
Charge low	1
High number adjacent charges	3
Low number adjacent charges	3
Intensity threshold scale	0.01
Min peak significance	1
Negative charge	True
Noise compensation	True
Noise rejection	95
Number of peak models	1
Peak model width scale	1
Quality score threshold	0
Relative abundance threshold	0
Target peak mass	20,000
Target peak shape left	2
Target peak shape right	2

Results and discussion

Reversed-phase ion pairing chromatography was performed on the oligonucleotides. The method scope was to clean up the sample from salt and other reagents and elute the target oligonucleotide and related impurities as single peak. Initial experiments focused on testing HFIP concentrations of 0.01, 0.1, 0.5, 1.0, and 2%. As seen in Figure 2, the HFIP concentration was incrementally increased from 0.01% to 2% to maximize oligo peak area and minimize HFIP adduction. The industry standard is 2%. For the ISQ EM mass spectrometer, it was found that the adduct abundance versus the maximum spectral intensity was the greatest at 0.1% HFIP, which yielded the largest maximum charge state intensity. This 20x reduction of HFIP usage has a notable cost-saving impact as well.

Using the optimal HFIP concentration of 0.1% and the LC method conditions presented in Table 2, the chromatographic overlays represented in Figure 3 were obtained. The results represented by the traces show the elution of the oligomers without the separation of impurities, such as the N-1, N-2, N-3, but removing all extraneous synthesizing reagents present during the oligomer synthesis. A failed synthesis was observed in chromatogram 1 [black] where the expected 10-mer peak is absent.

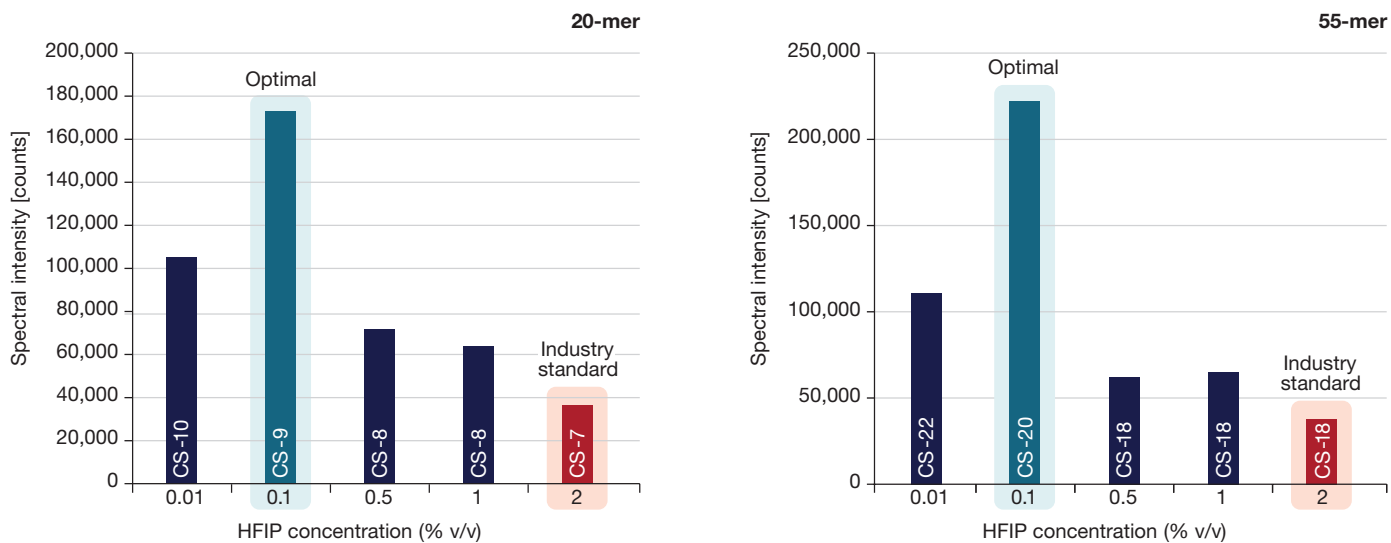


Figure 2. Impact of HFIP concentration on adduct abundance and signal intensity. The x-axis represents the five HFIP concentrations: 0.01, 0.1, 0.5, 1.0, and 2.0%. Different charge states [CS] provided the highest intensity. Displayed in the bar graphs are the respective maximum spectral charge state spectral intensity. The effect of the HFIP concentration was tested for the 20-mer and 55-mer.

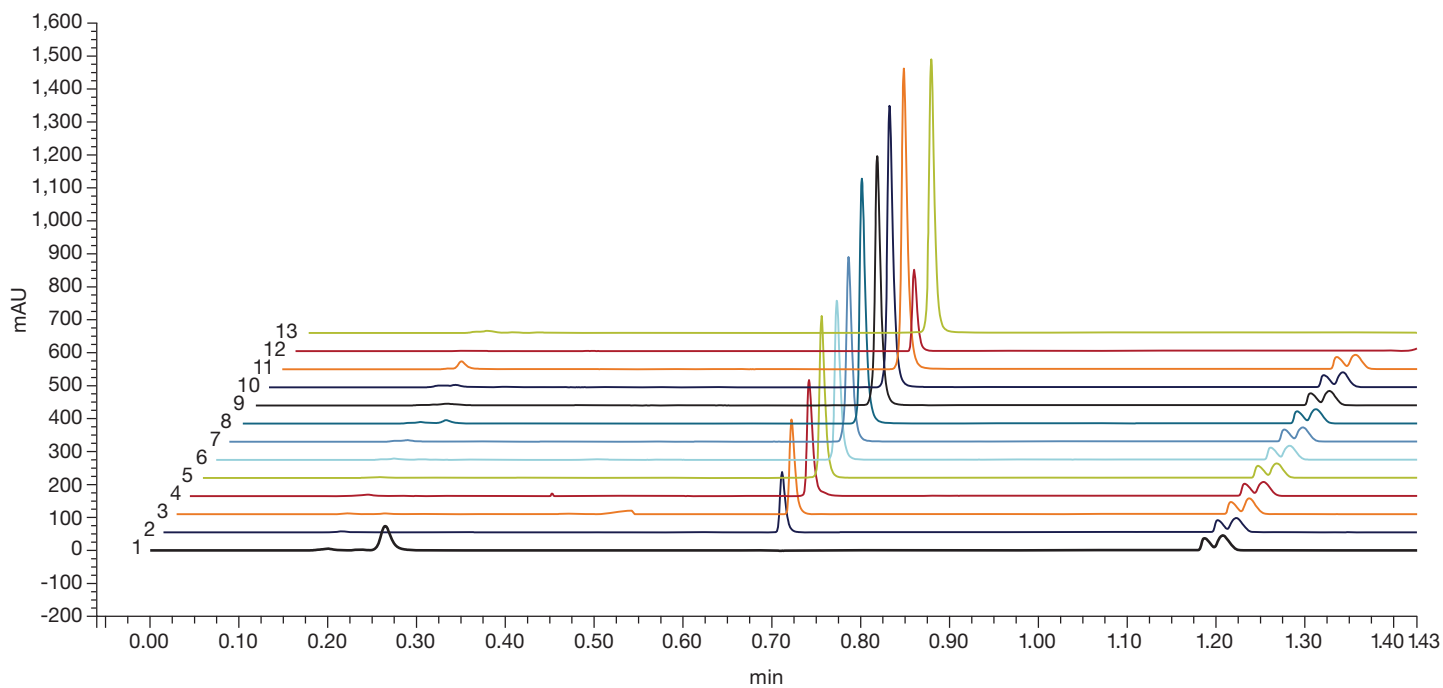


Figure 3. UV chromatograms for the oligomer array provided in Table 1, ranging from 10-mer to 60-mer. To be noted is the symmetry of the peaks although a stainless-steel flow cell was used.

Next, the ISQ EM source parameters were further optimized to provide the highest signal intensity of the maximum charge state for each oligomer while maintaining the lowest relative abundance of the HFIP adduct (Figure 4). The use of Custom Injection Variables in Chromeleon CDS (Figure 1) simplified the sequence, avoiding the generation of individual instrument methods for the changes to each ISQ EM source setting (for example, vaporizer temperature, ion transfer tube temperature, sheath gas/auxiliary gas pressure, and source voltage). One single method was used,

and the sequence table was extended to include the variables in Table 4. It was observed that the ion transfer tube temperature and the vaporizer temperature had the most significant impact on increasing spectral intensity of the maximum charge state, increasing spectral signal-to-noise (Figure 5), and minimizing HFIP adduct abundance. Thus, it was shown that the optimal MS conditions represented in Table 3 provided the highest spectral signal to the lowest adduct abundance for the oligonucleotide pair used for the optimization.

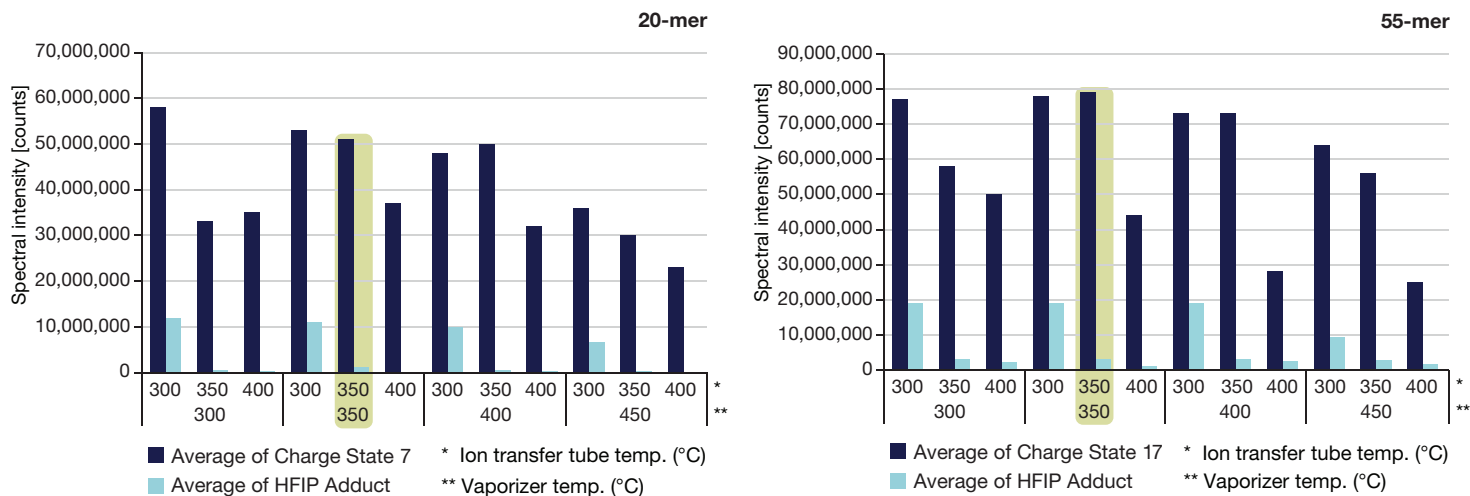


Figure 4. Optimization of source parameters continued. The source voltage and sheath gas/auxiliary pressure exhibited limited impact on the source optimization. -3,000 V source voltage and 75/7.5 psig were used in these experiments. The graph for the 20-mer shows the intensity of extracted ion chromatogram for charge state 7 and the neighboring HFIP adduct abundance. The 55-mer graph displays this same comparison but focusing on charge state 17.

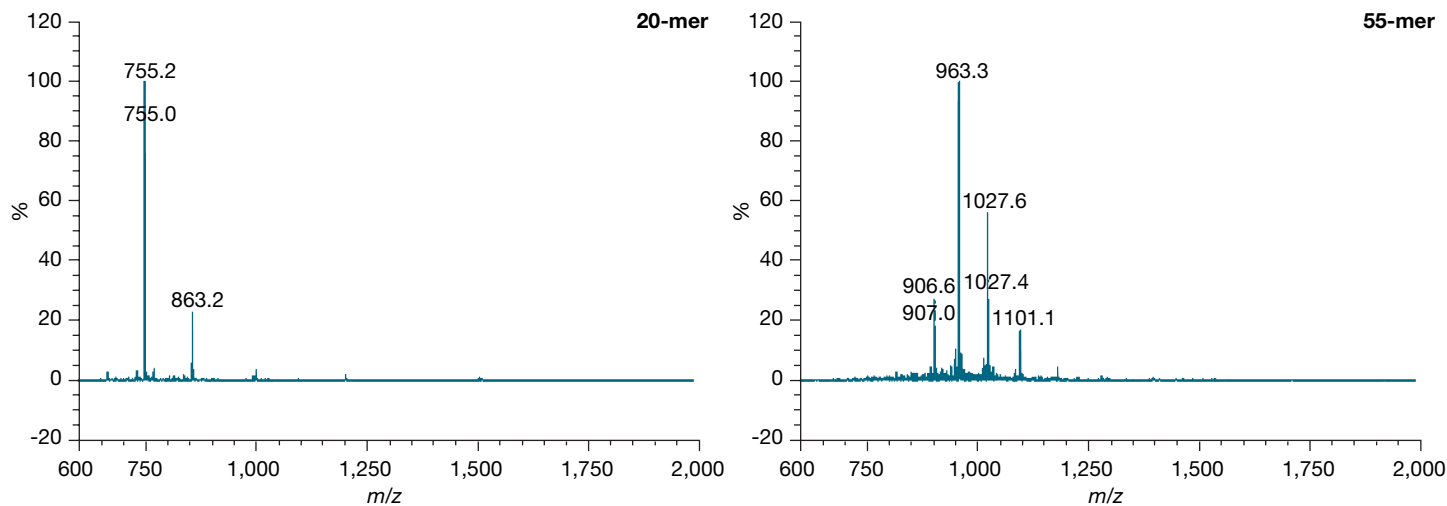


Figure 5. Spectral analysis of the 20-mer and 55-mer. Over the array of the oligomers, the spectral complexity increases with the length of the oligomer chain.

After the entire oligomer array was analyzed with the optimized HFIP concentration, LC method, and MS settings, data were analyzed using the intact mass deconvolution, mass confirmation, and report. Using the deconvolution settings (Table 5), oligomer array spectra were analyzed for their respective intact masses (Table 1). The measured intact mass was then compared to the expected mass. This was performed with the Custom Injection

Variables option where the expected intact mass of the target oligomer and target mass accuracy is defined by the user within the injection sequence (Figure 7). The confirmation that the measured mass matched the expected mass within the specified target mass accuracy was automatically visualized as a pass/fail result in the sequence report (Figure 8).

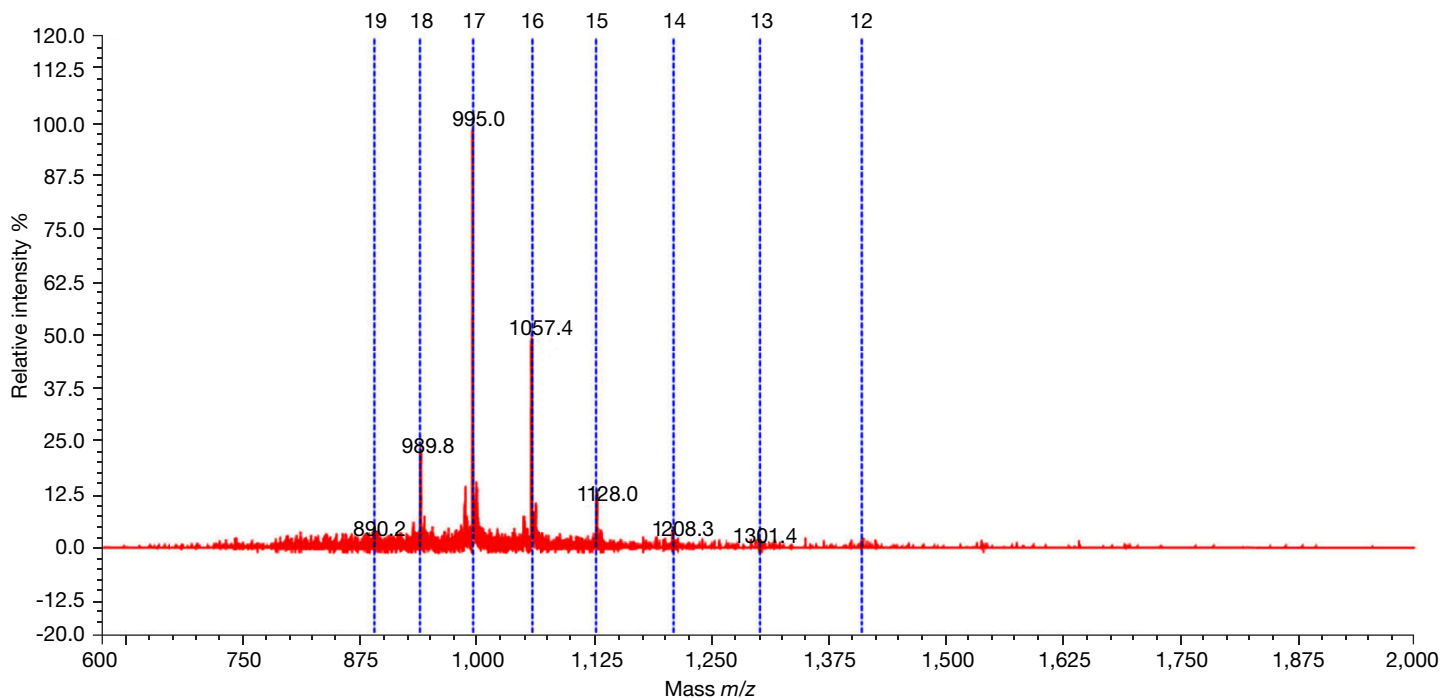


Figure 6. Example of intact mass deconvolution using the 55-mer (sample 13). The identified charge states are overlaid to the original MS spectrum.

Run Finished

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#	UV_VIS_1	Name	*ExpectedMass [Da]	*TargetAccuracy [Da]	Position	Volume [μl]	Instrument Method	Processing Method
14		Sample A11 - 15mer	4559.0	5	G:A3	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
15		Sample A11 - 15mer	4559.0	5	G:A3	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
16		Sample A11 - 15mer	4559.0	5	G:A3	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
17		Sample B08 - 20mer	6047.9	5	G:A4	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
18		Sample B08 - 20mer	6047.9	5	G:A4	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
19		Sample B08 - 20mer	6047.9	5	G:A4	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
20		Sample C08 - 25mer	7689.0	5	G:A5	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
21		Sample C08 - 25mer	7689.0	5	G:A5	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
22		Sample C08 - 25mer	7689.0	5	G:A5	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
23		Sample D06 - 30mer	9184.0	5	G:A6	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
24		Sample D06 - 30mer	9184.0	5	G:A6	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
25		Sample D06 - 30mer	9184.0	5	G:A6	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
26		Sample D11 - 35mer	10802.0	5	G:A7	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
27		Sample D11 - 35mer	10802.0	5	G:A7	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution
28		Sample D11 - 35mer	10802.0	5	G:A7	2.00	Oligo QC Method	Oligonucleotides ISQEM deconvolution

Figure 7. Expected Mass and Target Mass Accuracy. The Custom Injection Variables under the Custom Columns button in Chromeleon CDS allow the user to enter the expected target mass of the oligomer and define the target mass accuracy.

After the sequence of injections is completed, the mass of the oligonucleotides is visualized in the Chromeleon Report Template. The report table (Figure 8) clearly indicates if the expected mass is confirmed as the most abundant component (green text “Yes, Most Abundant”). When the expected mass is not confirmed

within the Target Accuracy limits (10 Da in the example case), the injection is marked by the red text “No Match”. An additional report result, not seen in this example, is the blue text “Yes, Other Component”, which indicates that the target mass is found but is not the most abundant deconvoluted component.

Inj. No.	Oligonucleotide Name	Position	Target Accuracy	Expected Mass	Matches IPD Component?	Measured Mass
			Da	Da		Da
1	1	G:A2	10.0	3206	No Match	12658.2
2	1	G:A2	10.0	3206	No Match	12951.8
3	1	G:A2	10.0	3206	No Match	8378.4
4	2	G:A3	10.0	4559	Yes, Most Abundant	4559.8
5	2	G:A3	10.0	4559	Yes, Most Abundant	4559.8
6	2	G:A3	10.0	4559	Yes, Most Abundant	4560.0
7	3	G:A4	10.0	6047.9	Yes, Most Abundant	6049.6
8	3	G:A4	10.0	6047.9	Yes, Most Abundant	6049.2
9	3	G:A4	10.0	6047.9	Yes, Most Abundant	6049.2
10	4	G:A5	10.0	7689	Yes, Most Abundant	7690.6
11	4	G:A5	10.0	7689	Yes, Most Abundant	7690.7
12	4	G:A5	10.0	7689	Yes, Most Abundant	7690.7
13	5	G:A6	10.0	9184	Yes, Most Abundant	9186.2
14	5	G:A6	10.0	9184	Yes, Most Abundant	9186.4
15	5	G:A6	10.0	9184	Yes, Most Abundant	9186.2
16	6	G:A7	10.0	10802	Yes, Most Abundant	10805.2
17	6	G:A7	10.0	10802	Yes, Most Abundant	10805.0
18	6	G:A7	10.0	10802	Yes, Most Abundant	10804.9
19	7	G:A8	10.0	12249.9	Yes, Most Abundant	12253.5
20	7	G:A8	10.0	12249.9	Yes, Most Abundant	12253.0
21	7	G:A8	10.0	12249.9	Yes, Most Abundant	12253.8
22	8	G:A9	10.0	13839.9	Yes, Most Abundant	13844.7
23	8	G:A9	10.0	13839.9	Yes, Most Abundant	13843.8
24	8	G:A9	10.0	13839.9	Yes, Most Abundant	13844.0
25	9	G:B1	10.0	15425	Yes, Most Abundant	15429.6
26	9	G:B1	10.0	15425	Yes, Most Abundant	15430.0
27	9	G:B1	10.0	15425	Yes, Most Abundant	15429.7
28	10	G:B2	10.0	16897.9	Yes, Most Abundant	16903.7
29	10	G:B2	10.0	16897.9	Yes, Most Abundant	16903.4
30	10	G:B2	10.0	16897.9	Yes, Most Abundant	16903.1
31	11	G:B3	10.0	18394.9	Yes, Most Abundant	18401.1
32	11	G:B3	10.0	18394.9	Yes, Most Abundant	18401.0
33	11	G:B3	10.0	18394.9	Yes, Most Abundant	18401.0
34	12	G:F1	10.0	6059.9	Yes, Most Abundant	6060.8
35	13	G:F2	10.0	16928.9	Yes, Most Abundant	16932.4

Figure 8. Report template. This report template confirms with an easy-to-read pass/fail result for the presence of the target mass. Red text “No Match”: expected mass does not match any of the five most abundant deconvoluted masses. Green text “Yes, Most Abundant”: expected mass matches the most abundant deconvoluted mass.

Conclusion

This application note provides a complete workflow for the analysis of oligonucleotides via a high-throughput robust LC method, intact targeted mass confirmation, and a user-friendly report confirming that the expected oligonucleotide has been synthesized. The following features are included with this workflow:

- Optimal ISQ EM spectra quality is observed with 0.1% HFIP, much below the concentration typically found in the literature of 2% HFIP. Therefore, it reduces the consumption of HFIP by a factor of 20. In the case that 192 samples are run per day, a year's savings could amount to over \$3,500 in HFIP consumption.
- Reduction of HFIP adducts and no sodium adducts are observed.
- Samples are collected directly from the DNA synthesizer and injected neat. No sample preparation is needed.
- The ISQ EM parameters have been optimized for oligomers in the range 10–60 chain lengths.
- Suggested deconvolution parameters provide for a reliable and automated recognition of the oligomer mass. For oligomers with mass outside the described range and/or different spectra quality, different parameters for the deconvolution method may be required.
- The entire workflow including the LC method, the sequence structure with Custom Injection Variables for expected mass and targeted mass accuracy, the MS settings, the deconvolution settings, and the reporting template is available for download as an [eWorkflow™ procedure in the Thermo Scientific™ AppsLab Library of Analytical Applications entry: High-throughput analysis of oligonucleotides using a single quadrupole mass spectrometer.](#)

Quality control laboratories screening large arrays of synthesized oligonucleotides can now, with a high level of confidence, easily confirm the quality of their oligonucleotide syntheses.

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