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

Dennis Köhler, Mauro De Pra
Thermo Fisher Scientific, Germering, Germany

ABSTRACT

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

Methods: Synthesized single length oligomers were analyzed without post synthesis purification. Thermo Scientific DNAPac RP (2.1x50mm, 4µm) was run on a Thermo Scientific™ Vanquish™ Flex Binary HPLC system with UV detection.

Results:
A step-by-step workflow including the analysis, deconvolution, and reporting of oligonucleotide synthesis quality control with a single quadrupole mass detector.

INTRODUCTION

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 Scientific™ offers a complete package consisting of the Thermo Scientific™ Vanquish™ Flex UHPLC using the Thermo Scientific™ DNAPac™ RP column for the separation. Determination of the intact oligonucleotide mass uses the 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 abundancy.

MATERIALS AND METHODS

Sample Preparation

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

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	TCTCCCGGACGGAAACCGCC	20	6047.9
4	AGGTAATTTCGCCTCATTGGGGGCC	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	ATAATCGAGAATTGGTATCGATTCGGGGCCACCCACAAGTCCGGTACACCAACCG	55	16897.9
11	CACACCTCGAAGAGTATTCCGTCCCGGAGCTGGTTAGGTGACTAACACTGCAAATTCTCT	60	18394.9
12	GGGGCGCTCTATCTTCCATC	20	6059.9
13	CCCGAGCGGAGTTTTGCGATAGTACACCAACCGAGCATCTCGAATTAAAGGCCTG	55	16928.9

Instrumentation

Thermo Scientific™ Vanquish™ Flex Binary HPLC System with Vanquish Variable Wavelength Detector F and ISQ EM single quadrupole mass detector.

Table 2. Chromatographic conditions

Column	Thermo Scientific DNAPac RP 2.1 × 50 mm, 4 µm (P/N 088924)
Flow rate:	0.70 mL/min
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 0.0 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 4.0 99 1
Column temperature:	70 °C, forced air mode 70 °C, active pre-heater
Injection volume:	2 µL
UV detector parameters:	λ=260 nm, 100 Hz

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

HESI Source Settings	
Vaporizer temperature	350 °C
Ion transfer tube temperature	350 °C
Source voltage	-3000 V
Sheath gas pressure	75 psig
Aux gas pressure	7.5 psig
Sweep gas pressure	0 psig

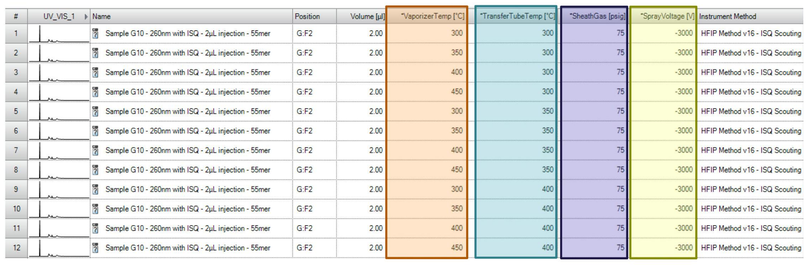
Scan Settings	
Mass range	600-2000 m/z
Dwell/Scan Time	0.5 s
Polarity	Negative
Spectrum Type	Profile
Source CID voltage	0 V

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 in Figure 1. 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

Figure 1. Inserted custom variables are as follows: VaporizerTemp (orange), TransferTubeTemp (blue), SheathGas (purple), SprayVoltage (yellow).



#	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

Chromatography Data System

Chromeleon 7.3 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 (IPD) 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.

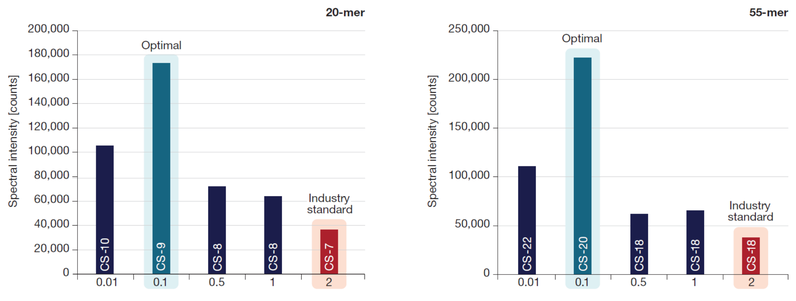
Table 5 Intact Protein Deconvolution settings

Parameter	Value	Parameter	Value
Peak retention window	0.7-0.8 min	**Low number adjacent charges**	3
Algorithm	ReSpect™	**Intensity threshold scale**	0.01
Output mass range	2000-20000 Da	**Min peak significance**	1
Deconvoluted spectra display mode	Isotopic Profile	**Negative charge**	True
Model mass range	2000-20000 Da	**Noise compensation**	True
Deconvoluted Mass Tolerance	100 ppm	**Noise rejection**	95
Peak model	Nucleotide	**Number of peak models**	1
Resolution	Raw File Specific	**Peak model width scale**	1
Charge carrier	H+	**Quality score threshold**	0
Charge high	30	**Relative abundance threshold**	0
Charge low	1	**Target peak mass**	20000
High number adjacent charges	3	**Target peak shape left**	2
		Target peak shape right	2

RESULTS

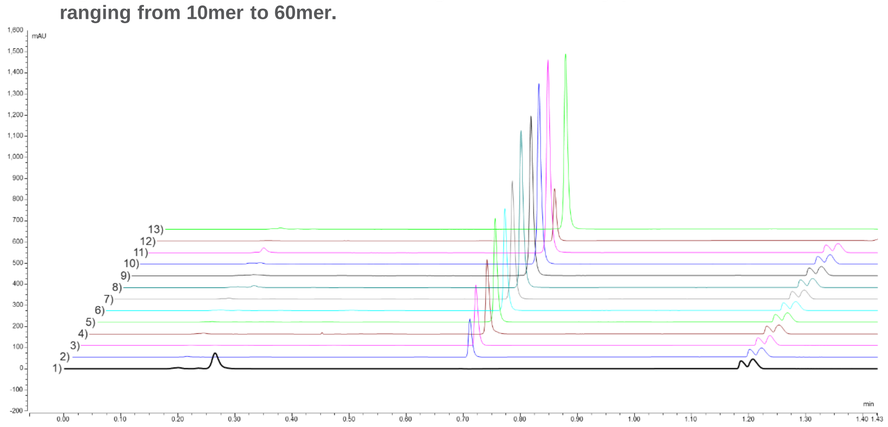
Reversed-phase ion pairing chromatography was performed on the oligonucleotides. The method scope was to clean-up the sample from salt and other reagents and elute the target oligonucleotide and related impurities as single peak. Initial experiments focused on testing HFIP concentrations of 0.01, 0.1, 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, it was found that the adduct abundancy versus the maximum spectral intensity was the greatest at 0.1% HFIP which yielded the lowest HFIP adduct relative abundancy and the largest maximum charge state's intensity. This 20x reduction of HFIP usage has a notable cost-saving impact as well.

Figure 2 Impact of HFIP concentration on adduct abundance and signal intensity.



Using the optimal HFIP concentration of 0.1% and given in the LC method conditions presented in Table 2, the following chromatographic overlays represented in Figure 3. 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, etc. but removing all extraneous synthesizing reagents present during the oligomer synthesis. One can observe that a failed synthesis occurred, like the 10mer seen in chromatogram 1 (black), where the expected oligomer peak is absent.

Figure 3 UV chromatograms for the oligonucleotide array provided in Table 1 ranging from 10mer to 60mer.



After the entire oligomer array was analyzed with the optimized HFIP concentration, LC method, and MS settings, data was analyzed using including 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 is performed with the Custom Injection Variables where the expected intact mass of the target oligomer and target mass accuracy is defined by the user within the injection sequence (Figure 5). 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 5).

Figure 4 Example of intact mass deconvolution using the 55-mer (sample 13). The identified charge states are overlaid to the original MS spectrum.

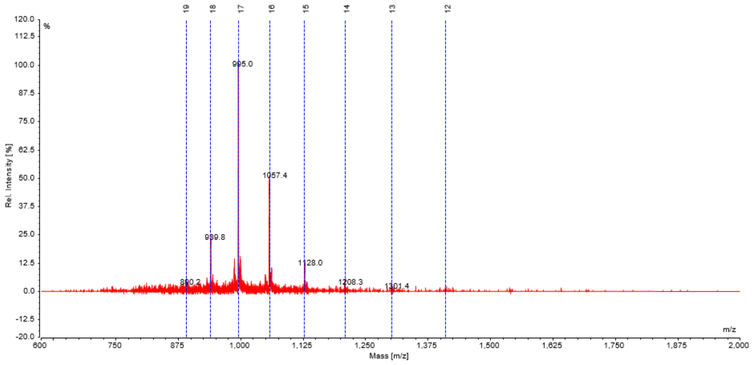
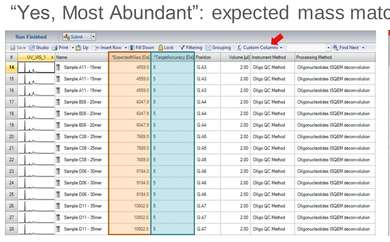


Figure 5 Expected Mass and Target Mass Accuracy with accompanying report. On the left using Custom Injection Variables in Chromeleon CDS allow the user to enter the expected target mass of the oligomer and define the target mass accuracy. This report template (right) confirms with an easy-to-read pass/fail 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.



Inj. No.	Oligonucleotide Name	Position	TargetAccuracy Da	ExpectedMass Da	Matches IPD Component?	Measured Mass 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

CONCLUSIONS

This work 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 expect 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.

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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