OligoQuest

Intuitive. Flexible. Automated. A modern workflow for oligonucleotide analysis

The growing importance of oligonucleotides in research, diagnostics and gene therapy, including tRNA, siRNA, and other modified oligos up to 120 bases, increased the regulatory interest in tools to verify their sequence as well as identify and quantify related impurities. LC-UV and LC-MS/MS are the preferred methods to characterize highly modified oligonucleotide sequences. QTOF instruments are widely utilized for that purpose as they can determine monoisotopic masses of intact oligonucleotides and their fragment ions with high mass accuracy. Here we describe a software, OligoQuest, that takes advantage of the data quality from high isotopic fidelity QTOF and timsTOF instruments and enables the fully automatic confirmation of sequences based on intact mass and MS/MS data as well as the quantitation and identification of synthesis by-products. The OligoQuest workflow in BioPharma Compass® is wizard driven to simplify the method setup and accommodates customer-defined sequence definitions to easily include the wide range of modified nucleotides used by the Pharma industry.

Challenges

Oligonucleotide by-products may include scrambled variants and Cytidine-to-Uridine conversions with no or +1 Da molecular weight differences to the target product sequence, respectively. These can be difficult to characterize solely based on their intact mass, information at the nucleotide level is required.

Oligonucleotide MS/MS spectra are complex; their manual interpretation is time-consuming and requires in-depth analysis skills, which renders the full analysis of several samples a day in the routine lab difficult and expensive.

Solution

The new OligoQuest workflow in BioPharma Compass provides an easy to learn tool for the interpretation of oligonucleotide MS and MS/MS spectra. The high isotopic fidelity of Bruker´s High-Resolution Accurate Mass (HRAM) QTOF and timsTOF instruments, together with the established SNAP algorithm for monoisotopic peak picking, provide reliable annotation of precursor and fragment ion masses. MS and MS/MS spectra are thereby matched with high certainty to sequence candidates such as the target sequence and expected by-products. OligoQuest works with user-defined sequence nomenclature thus avoiding a translation of lab-specific sequence definitions to a proprietary software format (Figure 1). Together with the proven workflow wizard and the simple reporting of results in BioPharma Compass, OligoQuest will help accelerate the characterization of DNA or RNA oligonucleotides towards the throughput required by the Pharma industry.

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Method

Data Acquisition

A 2´-permethylated RNA 24mer (Axolabs: # X87029) was diluted in Solvent A (1% HFIP, 0.23% TEA in H_2O) and separated using a 20- or 40-min gradient (Solvent B: 19.28% H_2O, 0.5% HFIP, 0.22% TEA in methanol) with an XBridge Oligonucleotide BEH C_{18}, 130 Å 2.5 µm, 2.1 x 50 mm column (Waters). The Elute HPLC was interfaced with the timsTOF Pro via the VIP-HESI ion source (all Bruker). MS/MS data were acquired in data dependent or MRM mode, offering flexibility for targeted and untargeted strategies. Both methods allow oligonucleotides to be fragmented by isolation of single or multiple precursor charge states depending upon sample complexity and experimental aims. Here, for autoMS/MS a 2 Hz acquisition method with a 1 sec cycle time was used which applied 40 eV collision energy for precursors at 500 *m/z*, ramping to 70 eV at 2000 *m/z*, with an isolation width of 3 *m/z*.

Data Analysis

Data were analyzed with OligoQuest. Monoisotopic peaks were picked from averaged chromatographic peak spectra using the SNAP algorithm (QF 0.5, SN 2). Sequences were written in a user defined syntax, which includes the building block definition and the separator between nucleotides (Figure 2). For each building block the fragment ions are defined automatically but can be adapted to user requirements in case of non-canonical fragmentation.

Results

The autoMS/MS spectrum in Figure 3 fully verified the expected sequence of the 24mer including its modifications based on 5´- and 3´-fragment ions. It was acquired from the dataset shown in Figure 4.

The intact mass analysis of the sample with sub-ppm accuracy confirmed the chemical formula and provided an overview of detected by-products (Figure 4) including a quantitative summary of the respective chromatographic peaks either based on the UV trace or the total ion chromatogram (TIC).

Spectrum Viewer | Building Block Editor

Open.. | Save | Save as.. | Active library: RNA-DNA.nt-json

Name	Alias	Sumformula	Base	Sugar	Phosphate	Mr (Mono)
Adenosine	A	C10H12N5O6P	C5H5N5	C5H10O5	H3O4P	329.053
Guanosine	G	C10H12N5O7P	C5H5N5O	C5H10O5	H3O4P	345.047
Deoxycytidine	dC	C9H12N3O6P	C4H5N3O	C5H10O4	H3O4P	289.046
Deoxythymidine	dT	C10H13N2O7P	C5H6N2O2	C5H10O4	H3O4P	304.046
Deoxyadenosine	dA	C10H12N5O5P	C5H5N5	C5H10O4	H3O4P	313.058
Deoxyguanosine	dG	C10H12N5O6P	C5H5N5O	C5H10O4	H3O4P	329.053
2'-O-methyl-Deoxycytidine	dc	C10H14N3O6P	C4H5N3O	C6H12O4	H3O4P	303.062
2'-O-methyl-Deoxythymidine	dt	C11H15N2O7P	C5H6N2O2	C6H12O4	H3O4P	318.062
2'-O-methyl-Deoxyadenosine	da	C11H14N5O5P	C5H5N5	C6H12O4	H3O4P	327.073
2'-O-methyl-Deoxyguanosine	dg	C11H14N5O6P	C5H5N5O	C6H12O4	H3O4P	343.068
2'-O-methyl-Cytidine	rm2C	C10H14N3O7P	C4H5N3O	C6H12O5	H3O4P	319.057
2'-O-methyl-Uridine	rm2U	C10H13N2O8P	C4H4N2O2	C6H12O5	H3O4P	320.041
2'-O-methyl-Adenosine	rm2A	C11H14N5O6P	C5H5N5	C6H12O5	H3O4P	343.068
2'-O--methyl-Guanosine	rm2G	C11H14N5O7P	C5H5N5O	C6H12O5	H3O4P	359.063
2'-O-methyl-Cytidine thioate	cs	C10H14N3O6PS	C4H5N3O	C6H12O5	H3O3PS	335.034
2'-O-methyl-Uridine thioate	us	C10H13N2O7PS	C4H4N2O2	C6H12O5	H3O3PS	336.018
2'-O-methyl-Adenosine thioate	as	C11H14N5O5PS	C5H5N5	C6H12O5	H3O3PS	359.045
2'-O-methyl-Guanosine thioate	gs	C11H14N5O6PS	C5H5N5O	C6H12O5	H3O3PS	375.04

Figure 1: Building Block Editor with example nucleotide definitions. Some of them originate from the MODOMICS database, some of them are user defined. The alias is used to define the sequences and to enable lab-specific nomenclature.

5OHgcacgcgugcuuuugcacgcgugc3OH	NONE
5OH rm2G rm2C rm2A rm2C rm2G rm2C rm2G rm2U rm2G rm2C rm2U rm2U rm2U rm2U rm2G rm2C rm2A rm2C rm2G rm2C rm2G rm2U rm2G rm2C 3OH	SPACE

Figure 2: The 24mer sequence. Two different versions of sequence syntax were used yielding identical results. The separator can be freely defined; here, no separator or a blank were used, respectively.

Figure 3: AutoMS/MS spectrum of the 24mer with fragment ion annotations in OligoQuest (A). The Sequence Map (B) displays the 5´- (red) and 3´-fragments (blue) including the observed ppm mass errors. The theoretical fragment ion masses including the matches with the spectrum are also displayed (C). The analysis is summarized in tabular form (D).

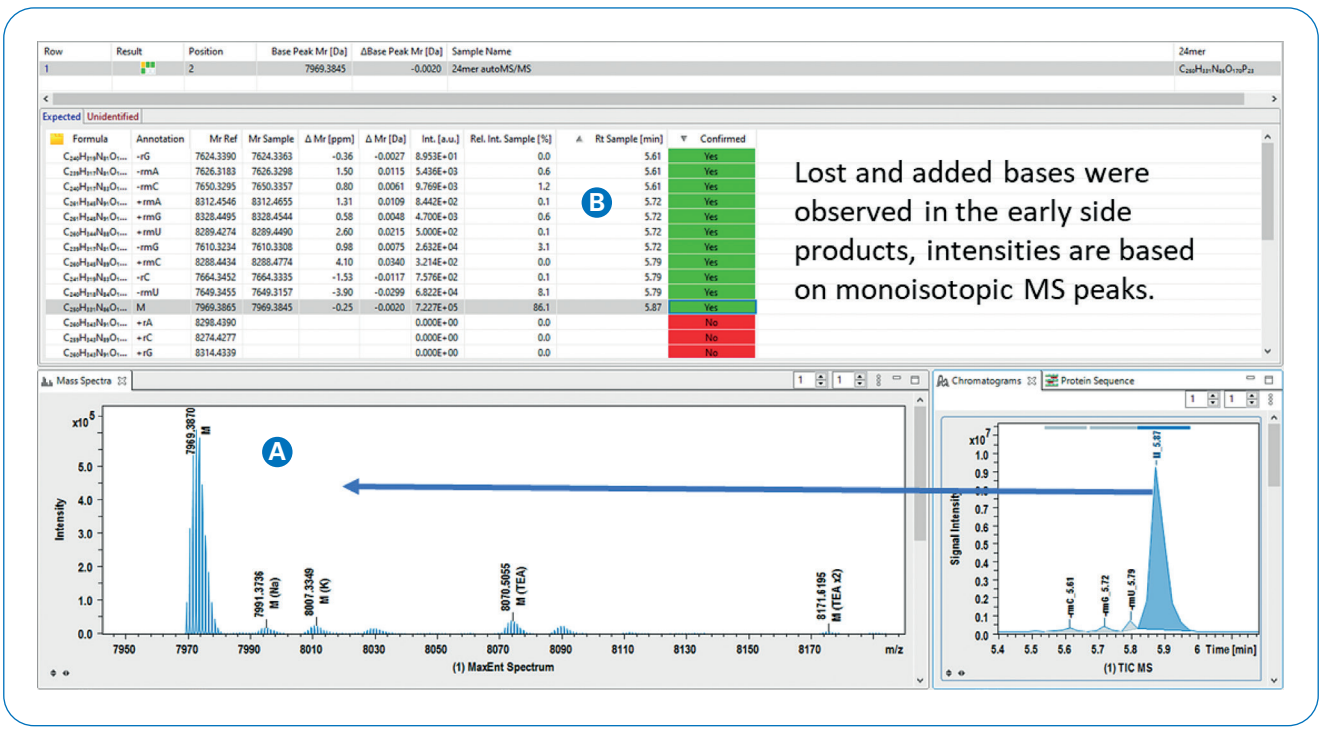

Figure 4: LC-MS analysis of the 24mer autoMS/MS dataset (A). The oligonucleotide was separated from all impurities, solely salt and ion pairing agent adducts were observed in the 24mer peak. Detected by-products are listed as well (B).

Butterfly | Multi Attributes | Chromatogram Peaks

Rt [min]	Annotation	Int.	Area	Rt Start [min]	Rt End [min]	Rel. Area [%]	Rel. Area BP [%]
5.61	-rmC_5.61	1.994E+05	4.714E+05	5.54	5.66	1.6	1.7
5.72	-rmG_5.72	2.852E+05	5.866E+05	5.67	5.77	2.0	2.2
5.79	-rmU_5.79	5.373E+05	1.234E+06	5.77	5.82	4.2	4.6
5.87	M_5.87	9.147E+06	2.695E+07	5.82	5.97	92.2	100.0

Figure 5: By-product LC-peak quantitation based on peak area was summarized in the Chromatogram Peaks table.

Conclusion

OligoQuest is a new analysis workflow in BioPharma Compass that automates the validation of oligonucleotide sequences using MS/MS datasets and quantitates by-products using LC-UV or LC-MS from the same dataset. It enables the analysis of complex oligonucleotide sequences which were previously extremely time-consuming and limited to experts. In addition, a lab-specific sequence syntax can be used, thus facilitating the insertion of the software into existing processes.

Customer Impressions

Dr. Giovanni Calderisi, Project Chemist QC, Bachem AG:

"The maXis II instrument with HRAM (High Resolution Accurate Mass) and isotopic fidelity is essential for our sophisticated elucidation capabilities in the context of oligonucleotide analysis, thus saving our team tremendous amounts of time. We have even been able to confidently analyze U/C conversions."

Director from a leading gene therapy company:

"The maXis II is a great instrument to support the development of gene therapy treatments. The same platform is suitable to characterize both lipids and guide RNAs. The isotopic fidelity observed for complex RNAs is essential to get a mass accuracy sufficient for unambiguous identity confirmation by intact mass and MS2."

Dr. Ingo Röhl, Managing Director Operations, Axolabs GmbH:

"Isomeric Oligos can be analyzed with OligoQuest allowing to identify base exchanges. Already the first version could be included in our day to day workflow."

Fritz Schweikart, Ph.D., Pharmaceutical Sciences/ AstraZeneca, Gothenburg:

"With OligoQuest we finally got a long, long waited evaluation tool in hands, that tremendously simplifies, if not even enables us to analyse MS/MS data in depth from our pharmaceutical ASO's. Chemical degradation now can be analysed at ease and manual investigation of MS/MS data is history. The user interaction with the tested beta version is remarkably easy and straightforward with respect to the complex mass matching algorithm's happening in the background."

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