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Auto MS/MS and targeted MS/MS in-depth qualitative and quantitative analysis of oligonucleotide synthesis products and side products

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Introduction

- Whilst confirmation of the full-length product (FLP) in oligonucleotide analysis is usually straightforward, characterization of low abundant side products can be more challenging
- We describe a method and software for highly specific and sensitive characterization of synthetic oligonucleotides, which combines UV-based quantitation of side products with automated MS/MS and subsequent targeted MSMS analysis.
- The approach can identify and quantify alterations of the FLP with high accuracy.

Methods

- A fully 2´-O-methylated RNA 24mer (mod3) was synthesized (Axolabs), annotated in the sequence as a, c, g and u.
- 0.4-1.6 µg were analyzed by LC-UV-QTOF MS and MS/MS using 2 scan modes:
- auto MS/MS datasets were used to quantify side products by UV and MS, and the sequences of the FLP, side products were identified by data dependent MS/MS (Fig. 1 and 2).
- Targeted MS/MS analyses were used to further increase sequence coverage and specificity for the identification of side products (Fig. 3).
- Datasets were analyzed by the OligoQuest workflow in the BioPharma Compass software (Bruker). Input were FLP sequences and respective datasets, output was the quantitative composition of the sample based on UV (260 nm) alone - or in combination with MS to address coeluting peaks.

Auto MS/MS analysis of the mod3 24mer FLP

Full sequence coverage was determined for the FLP. However, there is residual uncertainty for the a/g exchange variant – a16g being the more likely candidate.

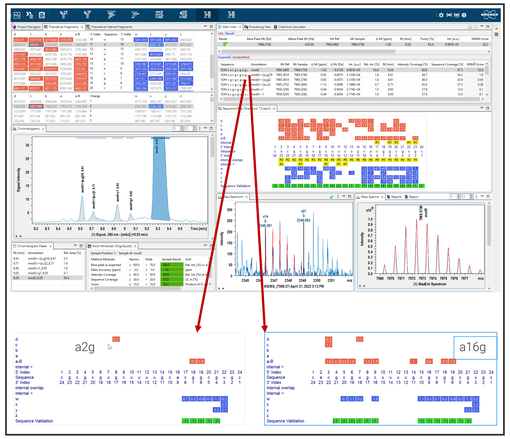

Fig. 1 Top; analysis overview of the FLP mod3, annotated MS and MS/MS peaks overlayed with theoretical isotope patterns. Bottom: Annotation of the variant sequences a2g and a16g

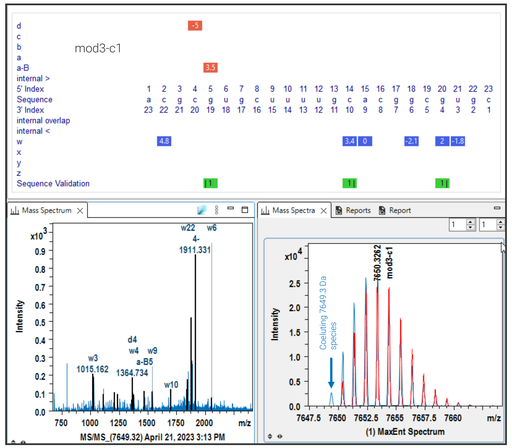

Fig. 2 Auto MSMS analysis of the mod3-c1 variant. The precursor ion isotope pattern (blue) (expected: red) indicates a coeluting species with -1 Da mass difference, the weak MS/MS spectrum was inconclusive.

Targeted MS/MS analysis of mod3-c1

The z=-4 ion of mod3-c1 was selected, and the targeted MSMS analysis yielded a much improved Sequence Map (Fig. 3, top) and an MS/MS score of 12.58 vs. 0.1 from the autoMSMS dataset. An even higher score was obtained for a mod3-c1 variant with an additional exchange of (u|c)9 resulting in an MS/MS score of 38.82 (Fig. 3, bottom) . This partial exchange of u9 to c9 also explained the isotopic pattern of the parent ion (Fig. 2) – c being 1 Da lighter than u.

Fig. 3 Targeted MSMS analysis of mod3-c1 provided a 56.5 sequence coverage (top). Another species, mod3-c1 (u|c)9, scored even higher (bottom), indicating a partial contribution of c9 instead of u9.

Auto MS/MS Analysis of the mod3-c1 23mer

The identification of the variant with loss of c1 was more challenging (Fig. 2). The isotope pattern of the precursor was heterogeneous and indicated the 78% presence of a coeluting 7649.3 Da species in addition to the 22% annotated mod3-c1 species. Quantitation was based on the accurate representation of the isotope pattern.

The MS/MS spectrum was weak and a follow up targeted MSMS analysis was undertaken to increase the quality of the MS/MS data.

Summary

- The analysis of the 24mer FLP mod3 allowed the detailed identification and quantitation of its side products using the timsTOF Pro2 and the OligoQuest workflows in BioPharma Compass.
- The result is a fully sequence-annotated FLP. Interactive tools allow to validate each residue in the sequence by fragment ion isotope pattern matching (Fig. 1).
- Some side products, such as the exchanges of (a|g) 16 and (a|c)2 (Fig. 1) were directly identified from autoMS/MS data at the 2% level; a mod3+g variant was detected (0.7%) largely based on MS data alone.
- In the autoMSMS workflow, the True Isotope Pattern capability of the orthogonal TOF analyzer allowed to safely detect and quantify a partial u-to-c conversion (-1 Da) by MS (Fig. 2).
- Targeted MSMS helped to localize the likely conversion site to (u|c)9 (Fig. 3).
- The high-quality isotope pattern of the precursor allowed to quantify the 8.93 min peak to consist of 78% mod3-c1(u|c)9 and 22% mod3-c1 – 2 species with just a 1 Da mass difference.

Conclusion

- Workflows were developed to identify the RNA full-length product (FLP) and quantify side products by LC-UV-MS.
- The workflows allow screening for FLP side products such as residue losses, exchanges, or additions, and to identify them.
- The True Isotope Pattern quality of the MS data enabled discerning a side product co-eluting with another one at a 1 Da mass difference.
- These OligoQuest workflows were implemented in the BioPharma Compass software for biopharmaceutical analysis.

Oligonucleotide side product characterization