Sequence Verification and Side Product Identification of Synthetic RNA Oligonucleotides by LC-ESI-PASEF and OligoQuest Software

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Introduction

Oligonucleotide characterization by mass spectrometry has gained significant interest recently with the increased use of DNA and RNA as research reagents as well as therapeutic molecules.

The manual interpretation of oligonucleotide MS/MS spectra for sequence verification is cumbersome and time-consuming.

We developed a workflow for the characterization of oligonucleotides using RP-UHPLC-ESI-PASEF (Parallel Accumulation Serial Fragmentation) or autoMS/MS, and the automatic assignment of the MS/MS fragment ions using a newly developed software, “OligoQuest” (*Fig 1*).

OligoQuest accepts any user-defined sequence syntax, simplifying the integration in existing processes.

We applied the workflow to an in-depth study of a synthetic permethylated RNA 24mer.

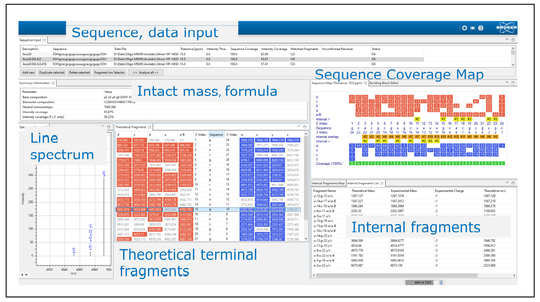

Fig. 1 OligoQuest user interface displaying 24mer analysis results. Sequences can be entered with a user-defined syntax. Result displays the sequence coverage map with all matching fragments. The terminal and internal fragment lists allow to verify matches in the charge deconvoluted line spectrum or in the profile spectrum.

Methods

The 24mer (Axolabs, #X87029) was 2´-methylated at every nucleotide; impurities were separated by UHPLC and analyzed using PASEF or autoMS/MS on a timsTOF Pro 2 (Bruker). In this experiment, multiple MS/MS spectra are accumulated for each charge state and the monoisotopic MS/MS peaklist is determined using the SNAP algorithm.

The OligoQuest software matches monoisotopic fragment ions against the ones calculated from the sequence - including modifications. It calculates 5´-, 3´- and internal fragment ions and provides a visual overview of the match (*Fig. 1*).

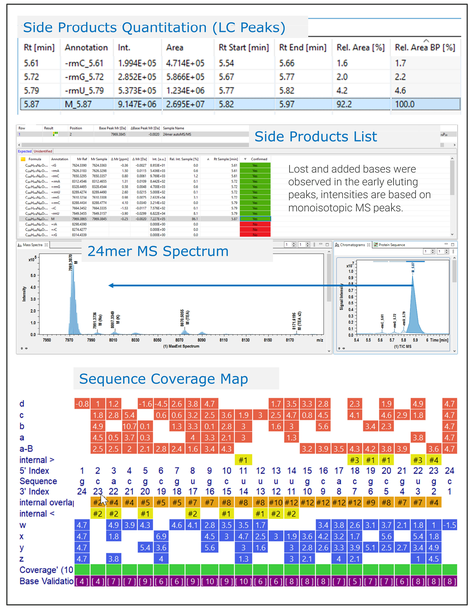

Fig. 2 RNA 24mer analysis: earlymers were quantified based on chromatogram peaks. 24mer MS spectrum itself did not show any side products – only salt and eluent adducts were present. MS/MS spectrum yielded 100% sequence coverage with redundancy in residue assignments.

In addition to the sequence verification of the 24mer, its impurities were characterized and quantified.

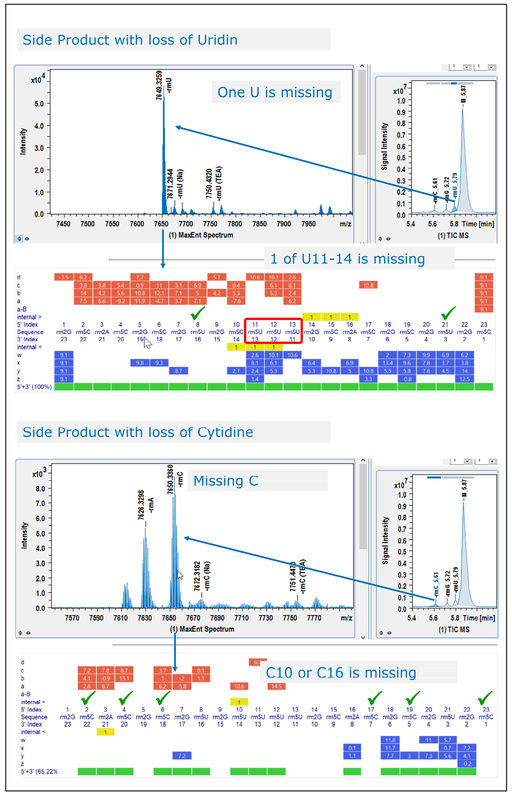

Fig. 3 Side product identification. Top: The U-deficient oligomer just contains 3 out of 4 core U residues.
Bottom: The loss of C was narrowed down to either C10 or C16 (see *Fig. 2*) – The presence of 6 other C-residues was confirmed.

Results

- The 24mer was purified and earlier eluting side products were quantified using the LC-peaks.
- The target RNA was MS/MS analyzed in the same dataset using OligoQuest yielding 100% sequence coverage (*Fig 2*).
- Individual side products with residue losses were detected and quantified based on intact mass.
- Side products with missing U or C were identified and the sites of losses located (*Fig 3*).

Summary

A workflow was established to confirm the intact mass and the sequence of synthetic oligonucleotides, including modifications.

Side products were characterized, and possible residue losses were localized.

OligoQuest allows to visualize the match between the charge deconvoluted monoisotopic peaklist and the sequence.

Matching fragment ions were also validated in the MS/MS profile spectra.

Free choice of sequence syntax is supported (compare *Fig 2 and Fig 3*).

Conclusions

- OligoQuest reduces the analysis time for oligonucleotide MS/MS spectra dramatically
- OligoQuest allows the confirmation of sequence candidates by automatic matching of MS/MS data and target sequences
- Free choice of sequence syntax facilitates adoption of the software
- Sequence variants can be qualified by their mass at high mass accuracy; the sites of variation can be located using MS/MS spectra and OligoQuest – a new workflow in BioPharma Compass
- The results can be verified either on the deconvoluted peaklist level or in the original profile spectra

BioPharma Compass