Optimized CID conditions for 24-75mer oligonucleotide MS/MS characterization

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Introduction

- MS/MS analysis of 20mer oligos is straightforward and typically sequences can be fully confirmed
- The task becomes increasingly difficult with oligonucleotides exceeding a range of approx. 30-40mers.
- In this work we established the collision energy (CE) for best fragmentation of oligonucleotides across a broad mass range, to achieve full sequence coverage (SC) by CID fragment ions.

Methods

2´-O-methylated RNA 24-60mers were synthesized in house (Axolabs). One µg each were injected to Elute UHPLC (Bruker) equipped with UV detector (Knauer) and 3 charge states were analyzed on a timsTOF Pro 2 mass spectrometer (Bruker) covering a broad range of m/z values. MS/MS spectra were analyzed with respect to the reduction of precursor intensity as a function of CE and the resulting sequence coverage (SC) (**Fig. 2**). The SC was automatically calculated in the BioPharma Compass software (Bruker).

Collision Energy Optimization

For the 24mer, the precursor ions m/z 796, 1992 and 2656 (z=-10, -4 and -3) were selected and MS/MS spectra acquired. The CE was increased to reduce the precursor ion intensity to 1% (CE 1%). This value was a function of the respective m/z and a linear relationship was established between m/z and CE: 30 eV (m/z 795) and 102 eV (m/z 2656) (**Fig. 1**). Analysis of the generated MS/MS spectra showed that a reduction of the CE by -10% yielded the best SC to verify oligonucleotide sequences. In addition, the higher abundant charge states tended to provide a higher SC compared to lower abundant ones.

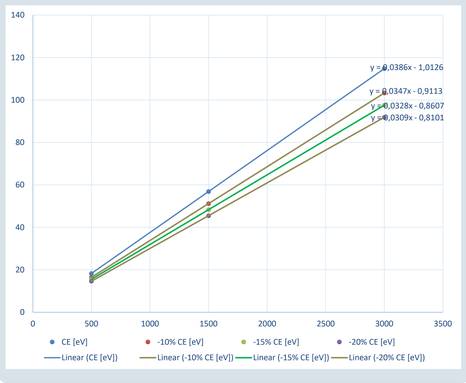

Fig. 1 Collision Energy (CE) as a function of m/z of the 24mer precursor ions. CE 1% (blue line) and the -10%, -15% and -20% lines are shown.

24-60mer Analysis

Oligos were all fragmented at CE -10% and 100% SC was achieved up to 40mers– the first residue commonly is not observed as a_1, w_1 fragments etc. are often not observed (**Fig. 2**).

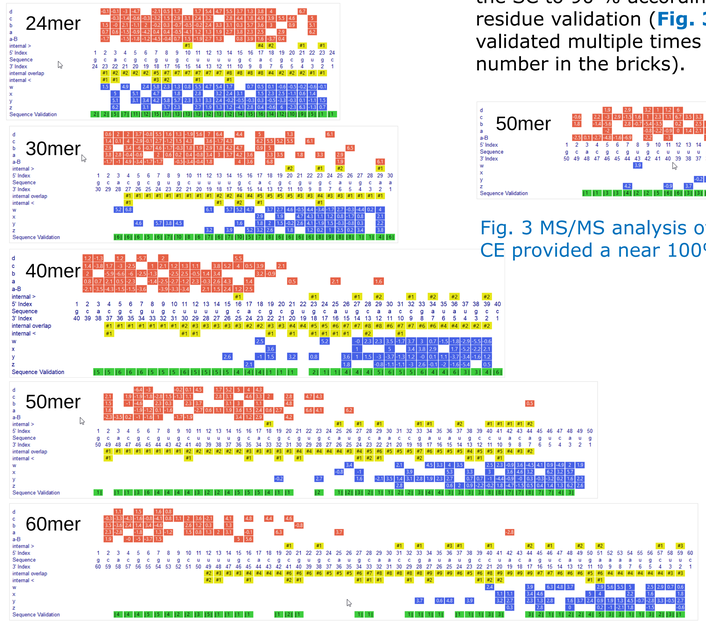

Fig. 2 MS/MS analysis of the 24-60mers at -10% CE. Terminal fragments are mapped to the respective sequences (5´-fragments red, 3´-fragments blue); internal fragments are shown in yellow.

50mer Optimized Analysis

In particular for 50 and 60mers the terminal fragments turned out to be too short to fully cover the sequence entirely. So the CE was reduced to -20% to increase the number of larger terminal fragment ions and to increase the SC to 90 % according to the green brick residue validation (**Fig. 3**) with most residues validated multiple times (indicated by the number in the bricks).

Fig. 3 MS/MS analysis of the 50mer at -20% CE provided a near 100% SC.

Summary

MS/MS conditions were established to routinely observe good sequence coverage for oligos from 24 -50 mers, with larger oligo fragmentation requiring relatively lower CE for high SC. 50mers are particularly relevant for the analysis of guide RNA (~100mers) as it can be cleaved enzymatically in the middle with engineered cleavage sites thus enabling their full sequence confirmation.

Conclusions

- timsTOF Pro and the OligoQuest workflow in the BioPharma Compass software enabled oligonucleotide sequence verification up to ~50mers
- CID conditions were established (27 - 90 eV for m/z 795 – 2656 ions) for optimal oligonucleotide sequence analysis in the 20-60mer range, based on terminal fragment ions
- For 50mers greater 90% SC was obtained under optimized acquisition conditions
- Full 50mer sequence validation is crucial for the analysis of gRNA used in methods like CRISPR-Cas9 based gene editing

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