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

Stuart Pengelley[1], Eckhard Belau[1], Yeni Yung-Mui[2], Dirk Wunderlich[1], Timo Schierling[3], Julia Schneider[3], Detlev Suckau[1]

[1]Bruker, Bremen, Germany
[2]Bruker, San Jose, CA, USA
3Axolabs, Kulmbach, Germany

Introduction to high quality oligonucleotide MS/MS analysis

- MS/MS analysis of 20mer oligonucleotides is typically straightforward where sequences can be fully confirmed.
- The task becomes increasingly challenging for oligonucleotides longer than 40mer.
- 50mers are of particular interest as guide RNA's (~100mers) can be enzymatically digested in the middle with engineered cleavage sites thus enabling their full sequence confirmation.
- In this work, we demonstrate optimized CID conditions that are applicable to fragmenting a wide mass range of intact oligonucleotides (24-60mers) with good sequence coverage.

Methods

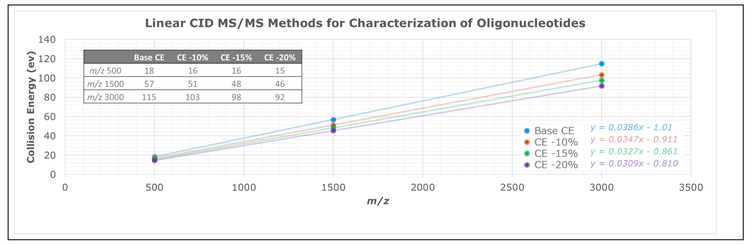
- Fully 2´-O-methylated RNA (24-60mers) were synthesized in-house (Axolabs).
- One µg of each oligonucleotide were injected onto a Bruker Elute UHPLC equipped with UV detector (Knauer) for subsequent autoMS/MS analyses on a Bruker timsTOF Pro 2 with a VIP-HESI ion source.
- MS/MS spectra of intact RNA were analyzed with respect to the reduction of precursor intensity as a function of collision energy and the resulting sequence coverage.
- Data was processed using the Bruker BioPharma Compass® 2023 software with the automated OligoQuest (autoMSMS) workflow wizard.

Collision energy (CE) method optimization

- A linear base MS/MS method, "Base CE", was first established for the fragmentation on the 24mer for three charge states (precursor ions) to 1% intensities (Fig. 1).
- Parameters from the Base CE method were further reduced, by 10-20%, and evaluated for MS/MS quality and sequence coverage (Fig. 1).

Table 1. For MS/MS spectra acquisition, the corresponding CE was applied to the 24 mer.

Charge State	Precursor Ion	Base CE	CE -10%	CE -20%
$[M-10H]^{10-}$	m/z 796	30	27	24
$[M-4H]^{4-}$	m/z 1992	76	68	61
$[M-3H]^{3-}$	m/z 2656	102	91	81



	Base CE	CE -10%	CE -15%	CE -20%
m/z 500	18	16	16	15
m/z 1500	57	51	48	46
m/z 3000	115	103	98	92

Figure 1. Graph of collision energy as a function of m/z. CE values for each method are listed in the inset table.

Results for larger oligonucleotides

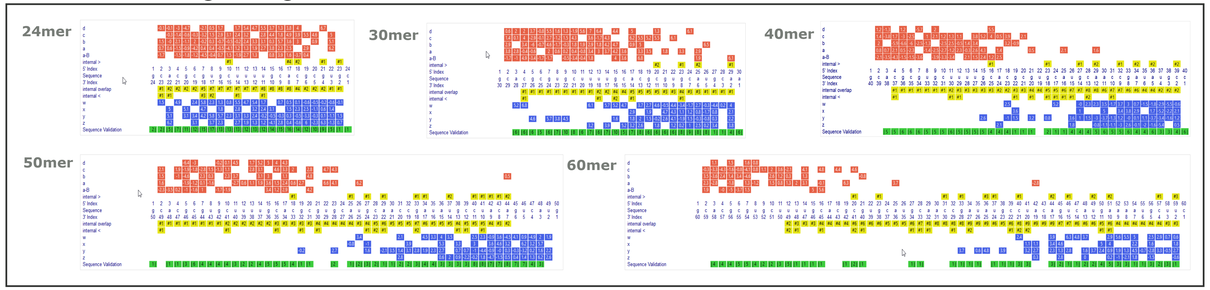


Fig. 2 Sequence maps of 24-60mer from MS/MS analyses at reduced Base CE by 10%. For SC calculations, both terminal and internal fragments are included- up to 100% SC was calculated for 24-40mers, and greater than 70% SC for the 50-60mers.

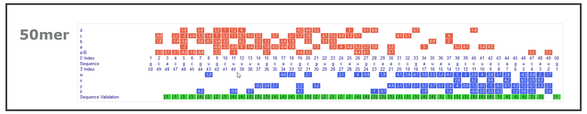


Figure 3. Sequence Map of 50mer from MS/MS analysis at reduced Base CE by 20%. 94% SC was calculated based on terminal fragments only.

- In addition to SC percentage, sequence maps (Fig. 2 and 3) provide an overview of MS/MS quality based on how well the data matches with the expected sequence.
  - In colored bricks, mapped 5´-fragment ions are highlighted in red, 3´-fragment ions in blue, with mass errors (in ppm) displayed. Total number of internal fragment ions are also displayed and highlighted in yellow.
  - The Sequence Validation, highlighted in green with residue frame scores in the bottom row, gives insight to validation quality for each residue using algorithms common in genome assembly.

Summary

- MS/MS methods were optimized for oligonucleotide characterization (Fig. 1).
- The SC at the reduced Base CE by 10% resulted in greater than 70% for the 60 mer and up to 100 % for the 24mer (Fig. 2).
  - For the 24mer, the CE corresponded to 27 - 91 eV for *m/z* 196-2656 (Table 1).
  - Terminal fragments were too short to cover the sequence entirely for the 50mer and 60mer.
- Further reducing the Base CE by 20%, a greater number of larger terminal fragment ions are produced.
  - For the 50mer, these terminal fragments contributed to greater than 94% SC (Fig. 3).

Conclusions

- The combination of timsTOF Pro 2 with a VIP-HESI ion source and OligoQuest from BioPharma Compass render oligonucleotide sequence verification straight forward.
- CID conditions were established to routinely observe good sequence coverage for intact oligonucleotides from 24-60mers, with larger oligonucleotides requiring relatively lower CE for high sequence coverage.
- For the 50mer, 94% SC was obtained under these optimized acquisition conditions.
- For the analysis of sgRNA, used in methods like CRISPR-Cas9 based gene editing, full 50mer sequence validation is of importance for enzymatically digested sgRNAs.

Oligonucleotide Sequence Validation

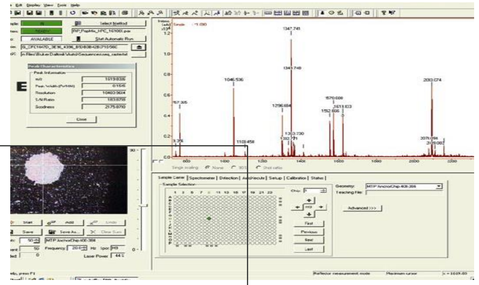


Fig. 3 Graphics Frame 49/51/49
Caption Roboto Light 20.5 pt, 5/65/105