ProtaGene

Rapid Analysis of Oligonucleotides by High-Resolution Accurate Mass CE-MS

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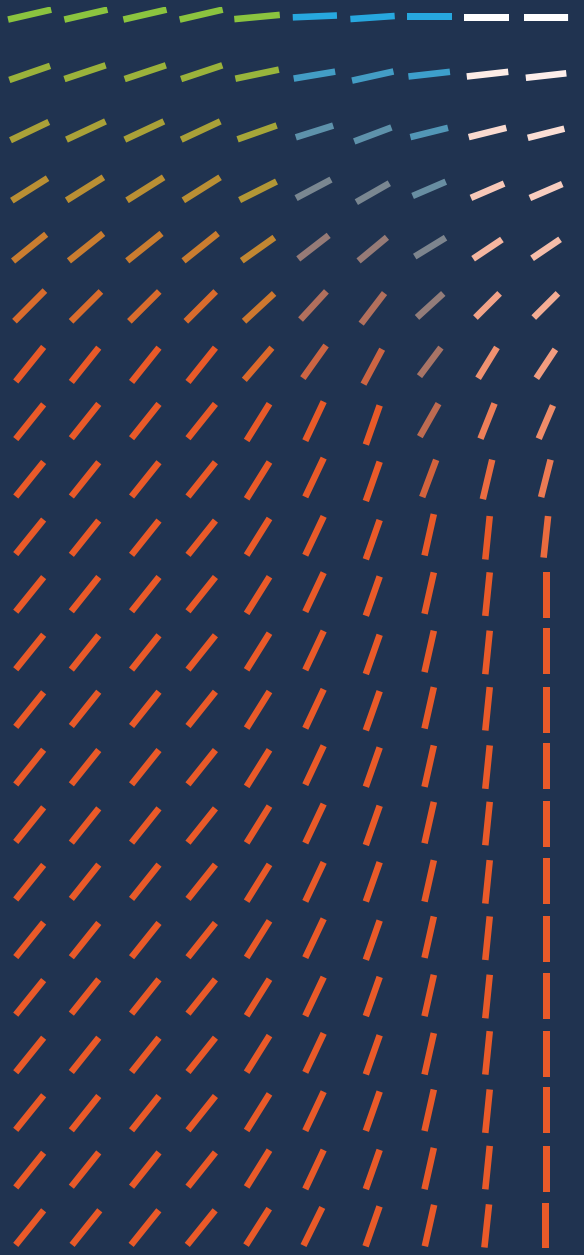
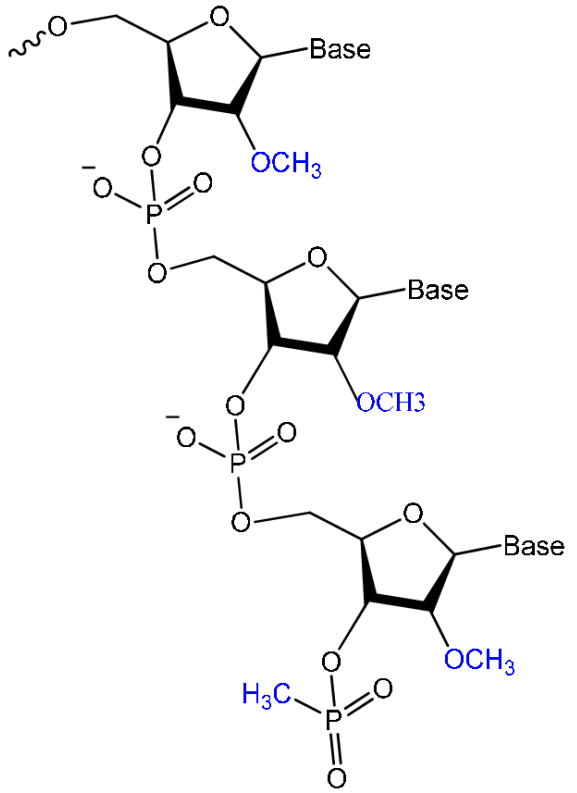


Oligonucleotides

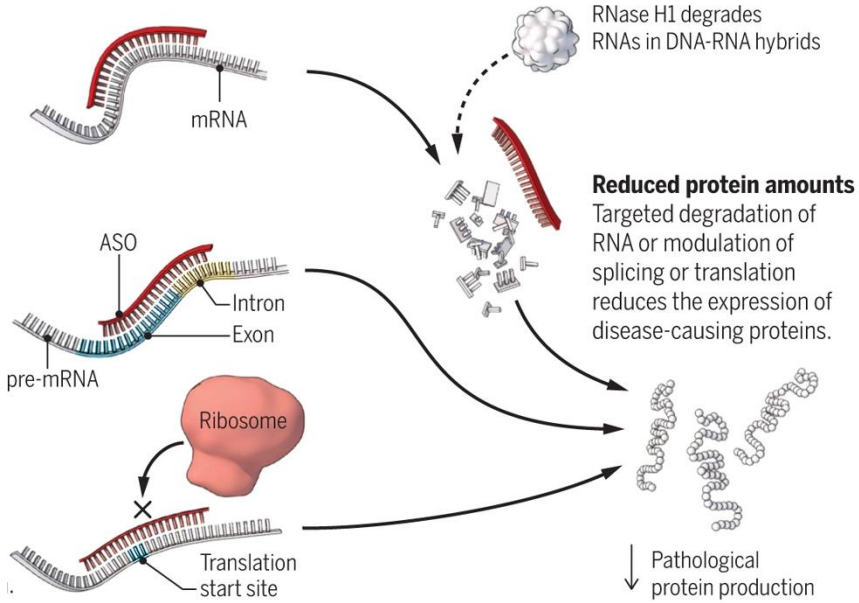
- Synthesized short DNA or RNA molecules (single or double stranded)
- Wide range of applications in molecular biology, biotechnology and therapy
- Oligonucleotides usually contain 10 to 30 nucleotides
- Classification of oligonucleotides:
  - Antisense oligonucleotides (ASOs)
  - Short interfering RNA (siRNA)
  - MicroRNA (miRNA)
  - Aptamers
  - CpG oligonucleotides



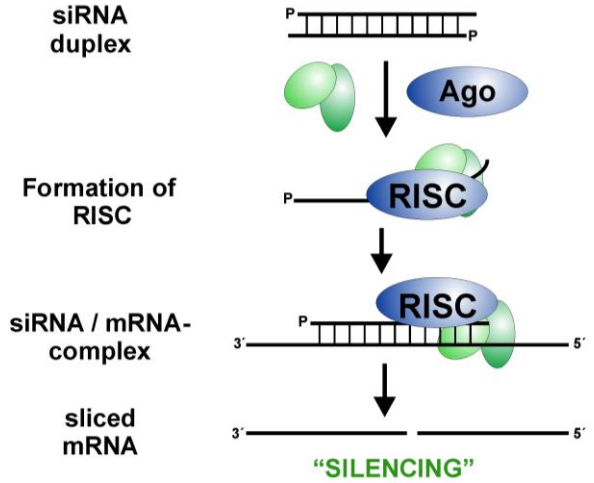
Odeh, F.; Nsairat, H.; Alshaer, W.; Ismail, M.A.; Esawi, E.; Qaqish, B.; Bawab, A.A.; Ismail, S.I. Aptamers Chemistry: Chemical Modifications and Conjugation Strategies. *Molecules* **2020**, *25*, 3. https://doi.org/10.3390/molecules25010003

Oligonucleotide Therapeutics

- Direct therapeutics against a variety of diseases:
  - Oncology, infectious diseases, metabolic disorders, genetic disorders, etc.
- Fifteen oligonucleotide therapeutics have been approved since 1998
  - The majority in the last five years
  - Many more are in development
- ASOs
  - ~18-30 nt, single stranded
  - Degradation of mRNA, suppression of transcription, inhibition of translation
- siRNA
  - ~19-23 nt, RNA duplex
  - Inhibition of translation, degradation of mRNA



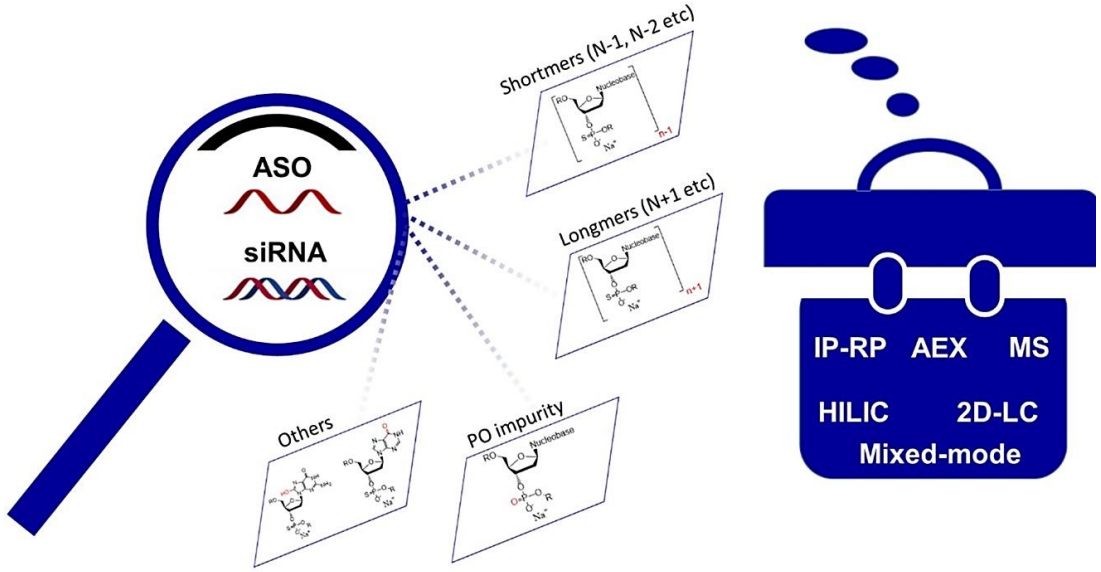
Leavitt, B; Tabrizi, S. Antisense oligonucleotides for neurodegeneration, *Science* **2020**, 367 (6485), 1428-1429. DOI: 10.1126/science.aba4624



http://www.uni-konstanz.de/FuF/chemie/jhartig/

Quality Control

- Confirmation of oligonucleotide mass and sequence
- Quantification of purity level
- Product- and process-related impurities determination and quantification:
  - Addition sequences (longmers, n+1)
  - Deletion sequences (shortmers, n-1, most common)
  - Phosphodiester analogs
  - Depurinated sequences
  - Modifications



Goyon, A; Yehl, P; Zhang, K. Characterization of therapeutic oligonucleotides by liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis* **2020**, 182, https://doi.org/10.1016/j.jpba.2020.113105

Capillary Electrophoresis using ZipChip®

- Capillary Zone Electrophoresis (CZE) separation
- Electrophoretic mobility (function of the charge-to-size ratio)
- Separation on a High-Resolution Bare Glass chip
- No ion-pairing agents required
- Low sample amounts needed (injection of 1 nL)
- Fast analysis (<10 min)

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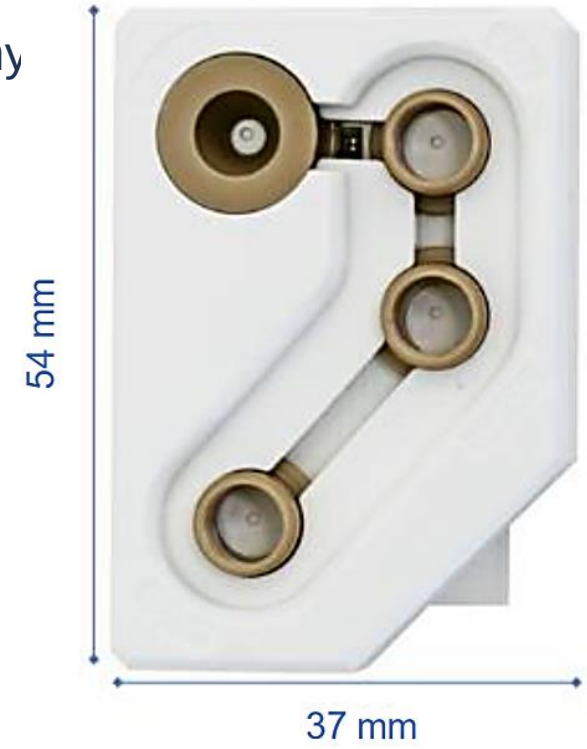
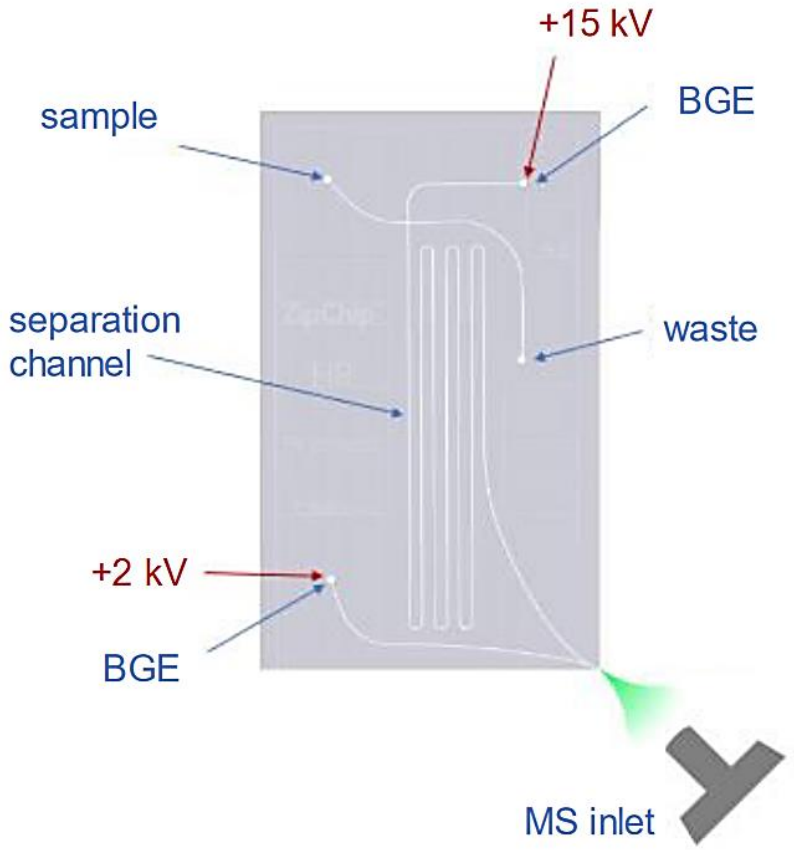
The ZipChip®

- Single piece of glass
- Microfluidic channels by photolithography and wet chemical etching
- Three major functional elements:
  - sample injection
  - electrophoretic separation
  - electrospray ionization

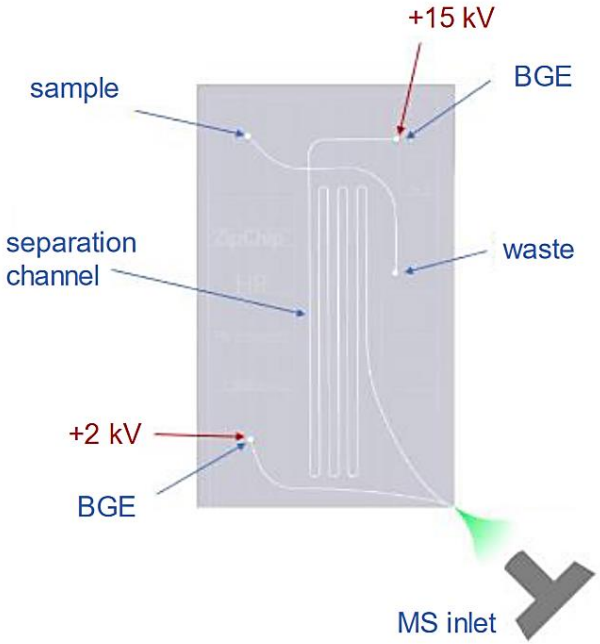


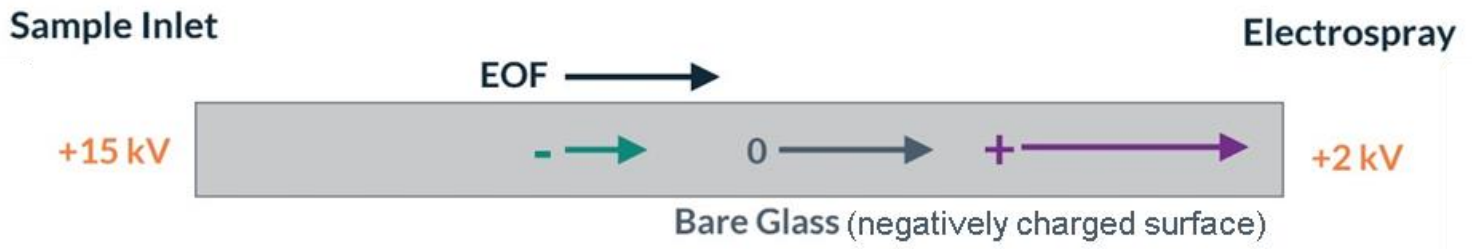


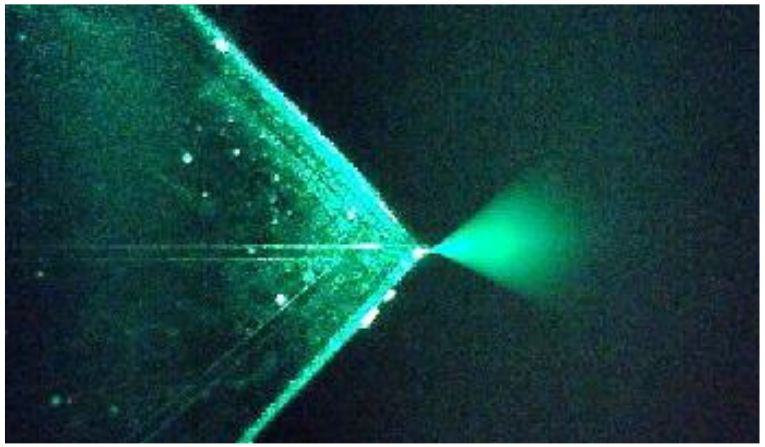
Tech Note 1.0 ZiChip What they are and how they work. 908devices

CE Separation of Oligonucleotides using ZipChip®

- Following the sample injection, voltage is applied to the BGE channels
- These voltages dictate the electrical field strength for the CE separation and the ESI voltage
- Analytes migrate down the separation channel in the electric field toward the ESI emitter
- Negatively charged oligonucleotides are carried to emitter with EOF







Tech Note 1.0 ZiChip What they are and how they work. 908devices

Capillary Electrophoresis Mass Spectrometry (CE-MS)

- Direct coupling to the mass spectrometer
  - Positive ESI mode
  - Stable nano-spray with high sensitivity
  - High-resolution accurate mass (HRAM) (60k resolution)
- Data evaluation with BYOS® Oligos
  - Identification (via MS/MS)
  - Quantification (peak integration)

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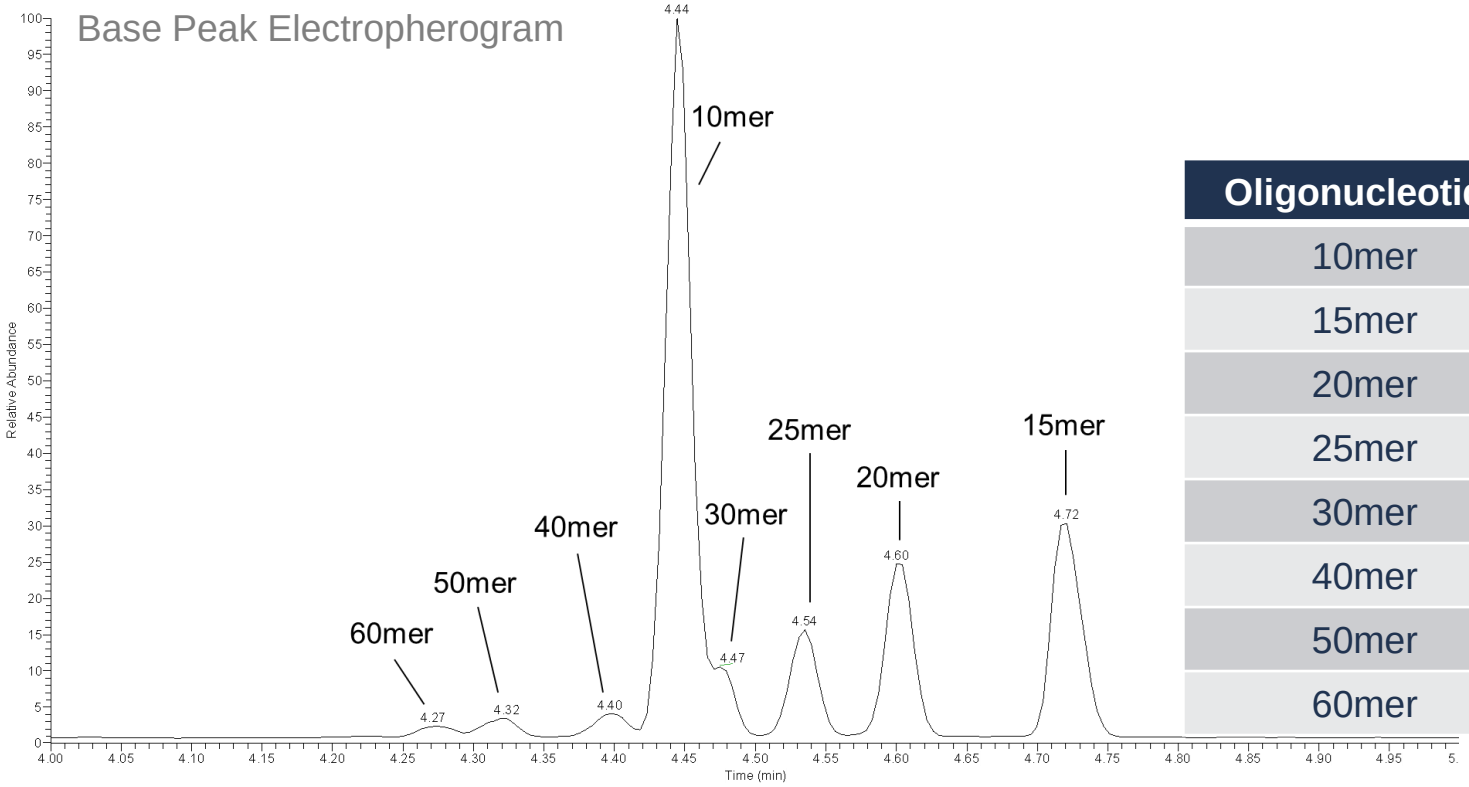
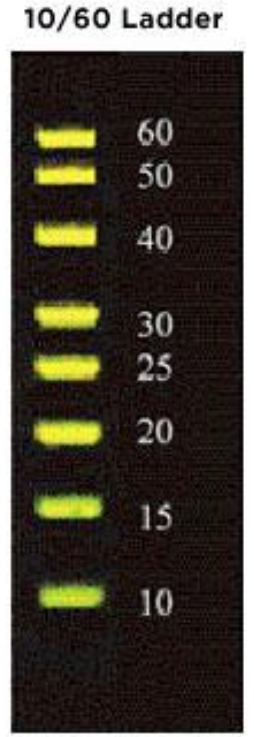
CE-MS of Oligonucleotides
Method Development

Separation of Oligonucleotides by CE-MS

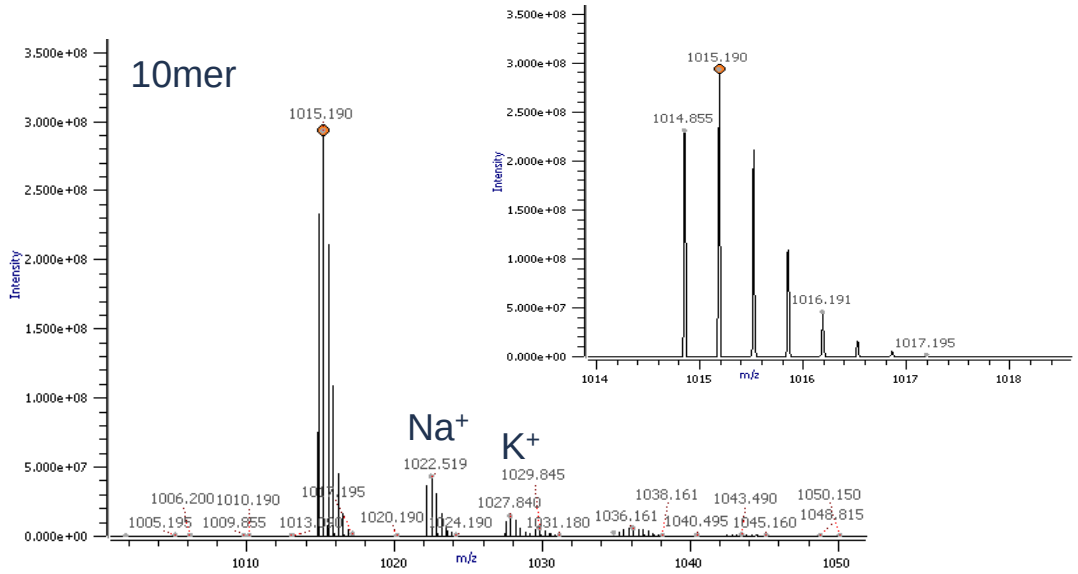
- DNA oligo length standard 10/60 Ladder (ssDNA, Integrated DNA Technologies)
- HRAM CE-MS analysis in a single-shot experiment
- The migration order ranged from low to high molecular weight, with the exception of the 10mer
- Correlating with the molarity, the 10mer showed the highest intensity

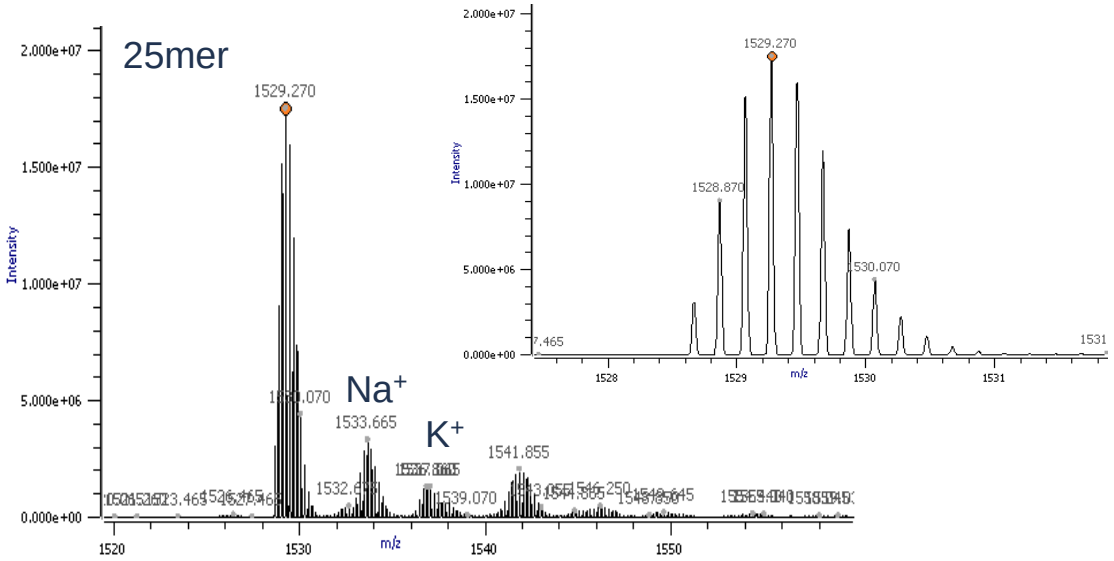


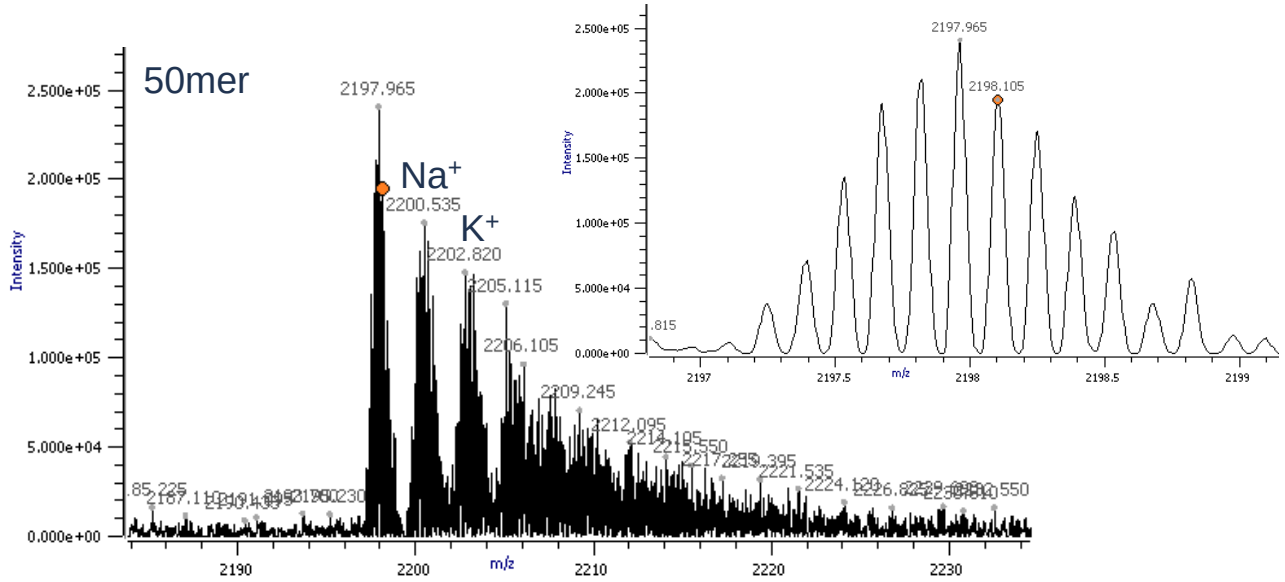
Oligonucleotide	Molecular Weight [Da]
10mer	3,043
15mer	4,634
20mer	6,117
25mer	7,642
30mer	9,191
40mer	12,274
50mer	15,379
60mer	18,493



High-Resolution Mass Spectra







- One to two charge states per oligonucleotide
- $[M+3H]^{3+}$ to $[M+7H]^{7+}$ species were the dominant charge states
- Isotope-resolved mass spectra
- Monoisotopic masses
- Alkali-metal ion adducts such as sodium and potassium were detected

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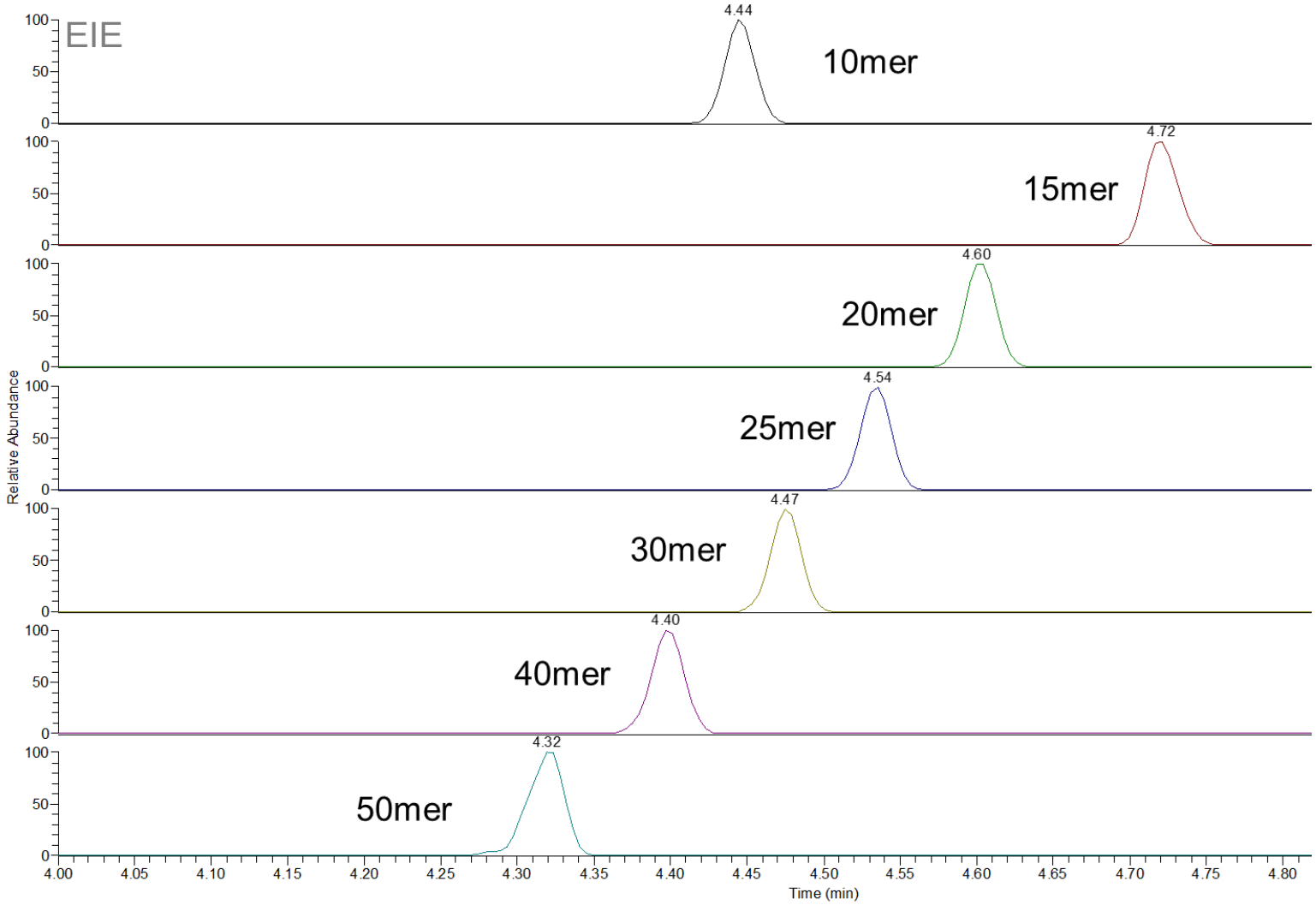
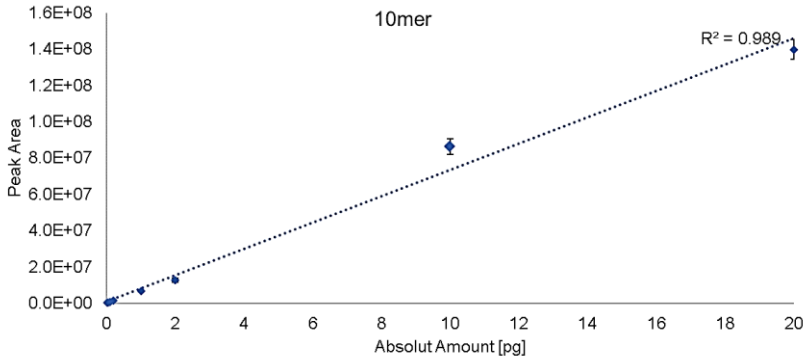
Benefits of HRAM-MS

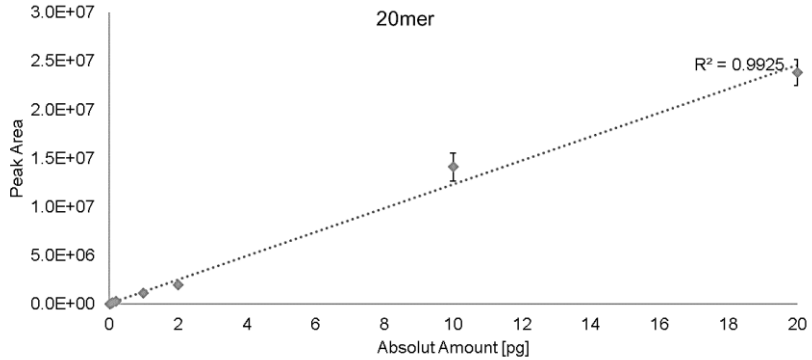


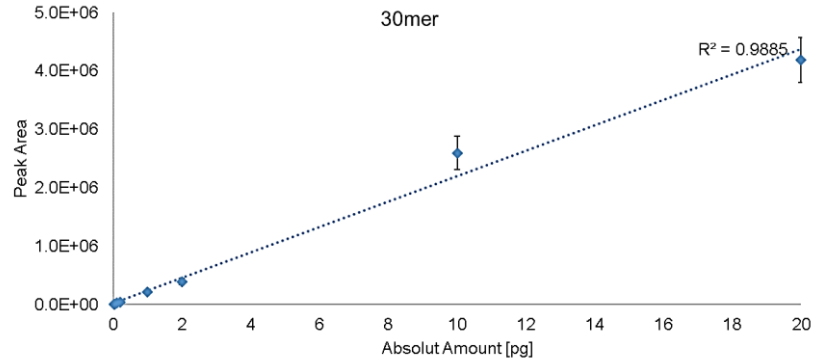
- HRAM-MS led to high specificity and a low mass deviation
- Unambiguous assignment of MW based on monoisotopic mass
- Quantification with high sensitivity by extracted ion electropherogram (EIE)
  - Most abundant charge state
  - Highest abundant isotope

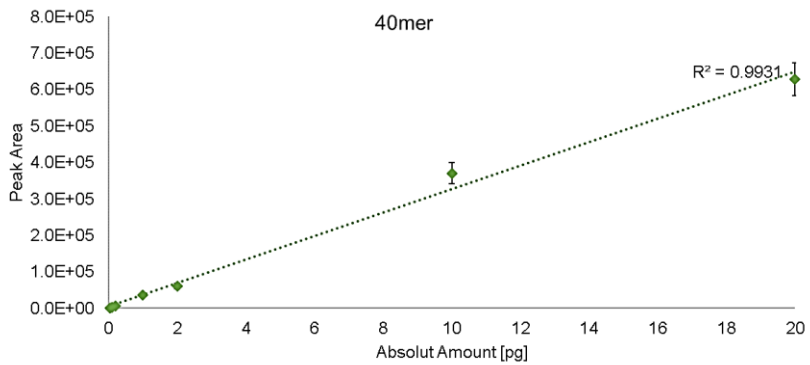
Quantification of Oligonucleotides

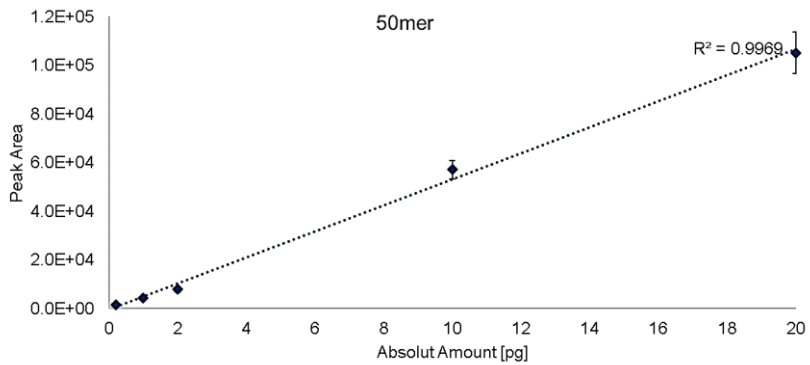
- Dilution series from 0.02 pg (1.1-6.6 nM) to 20 pg (1.1-6.6 µM)
- The quantification was linear over several orders of magnitude











Limit of Quantification

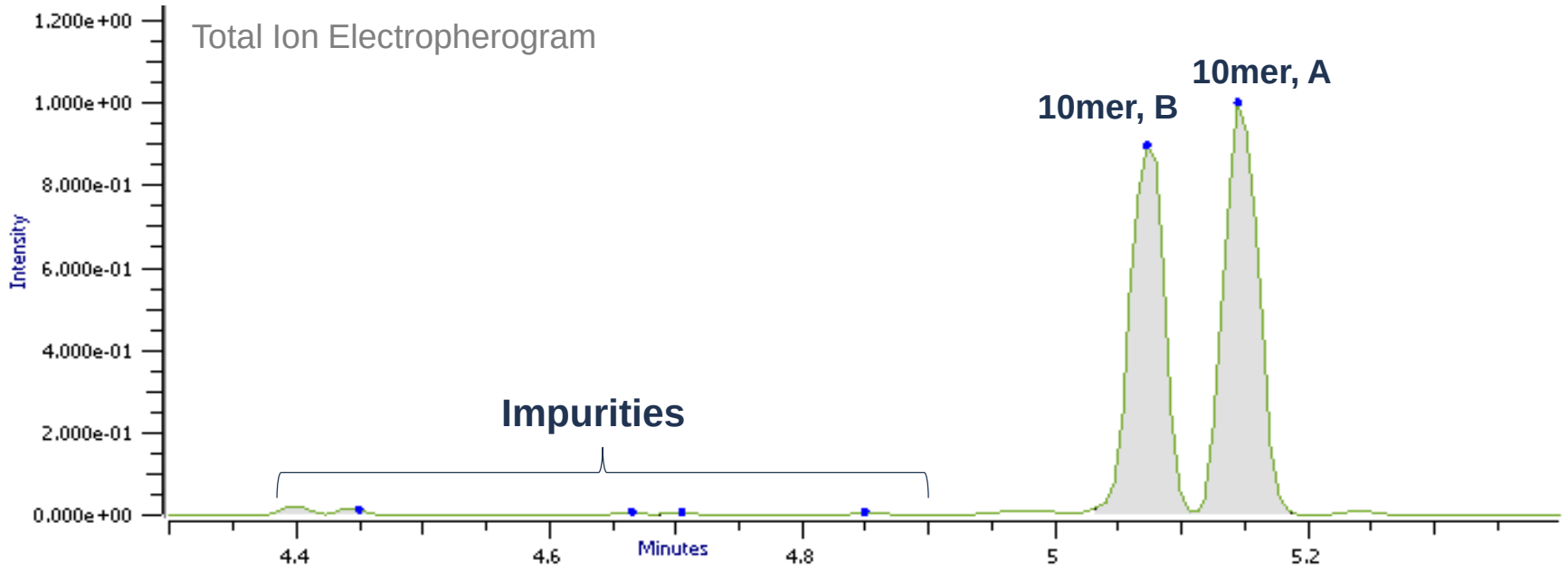
- LOQ within the low nM range
- Not reached for smaller oligonucleotides (10-30mer)
- CE-MS showed a high sensitivity

Oligonucleotide Length	Molecular Weight [Da]	Absolute amount [pg]	Concentration [nM]
10	3,043	<0.02	<6.49
15	4,634	<0.02	<4.32
20	6,117	<0.02	<3.27
25	7,642	<0.02	<2.62
30	9,191	<0.02	<2.12
40	12,274	0.05	4.07
50	15,379	0.20	13.00

CE-MS Analysis of Synthesized Oligonucleotides

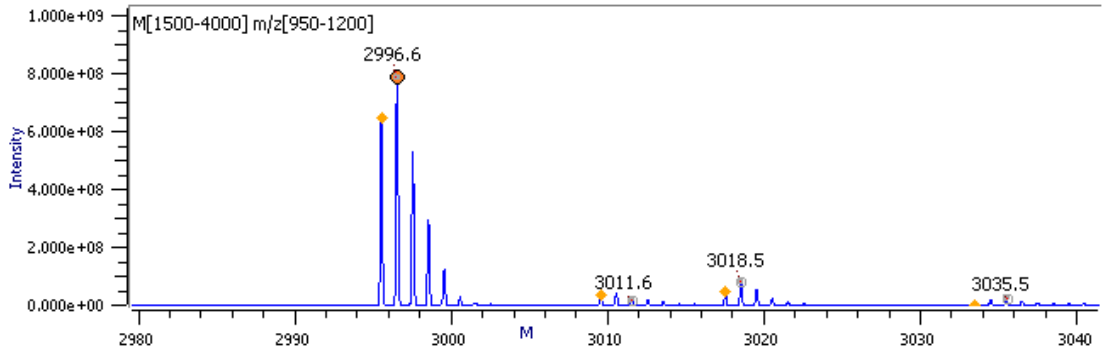
- Two individually synthesized 10mers (not purified) were measured
- Data evaluation with BYOS® Oligos
- The 10mers showed baseline separation
- Multiple impurities were detected

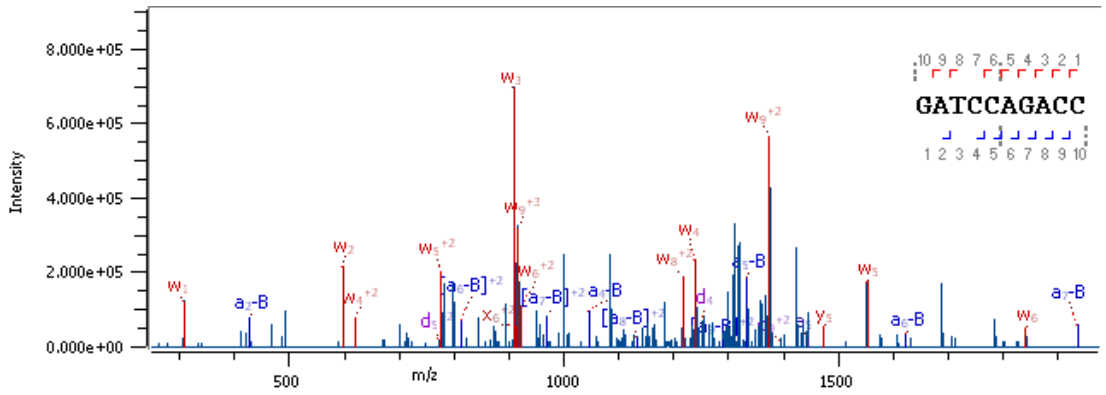
Name	Sequence	Molecular Weight [Da]
10mer,A	CAAAGCGACG	3,046
10mer,B	GATCCAGACC	2,997



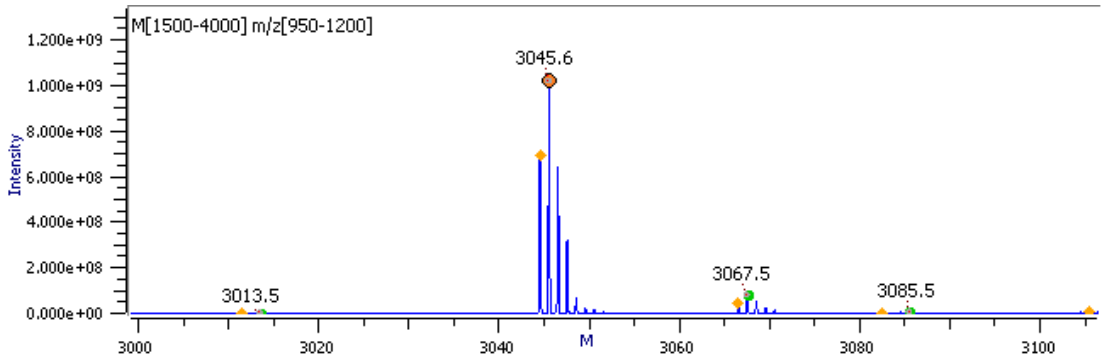
Sequence Confirmation by MS/MS

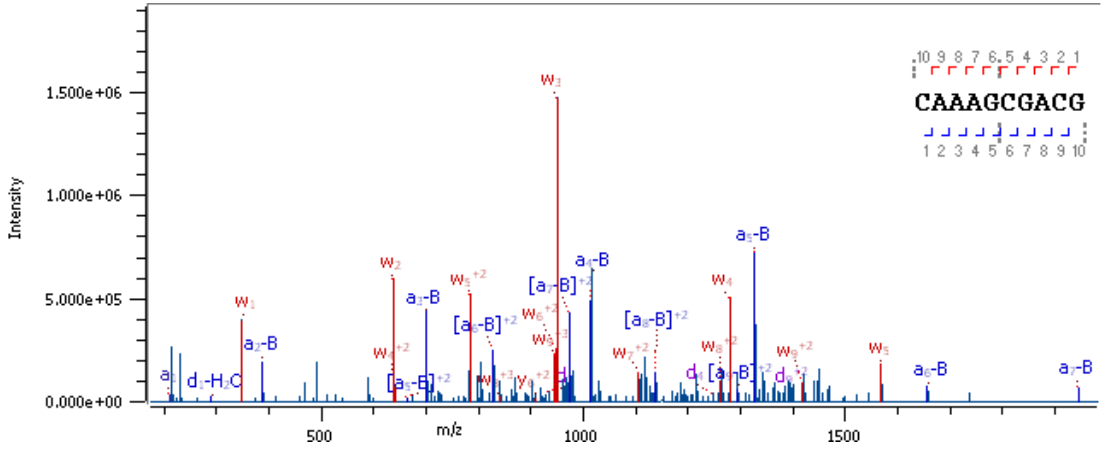
10mer,B





10mer,A





- The oligonucleotides were identified based on precursor mass and high quality MS/MS spectra
- High ion series coverage was achieved

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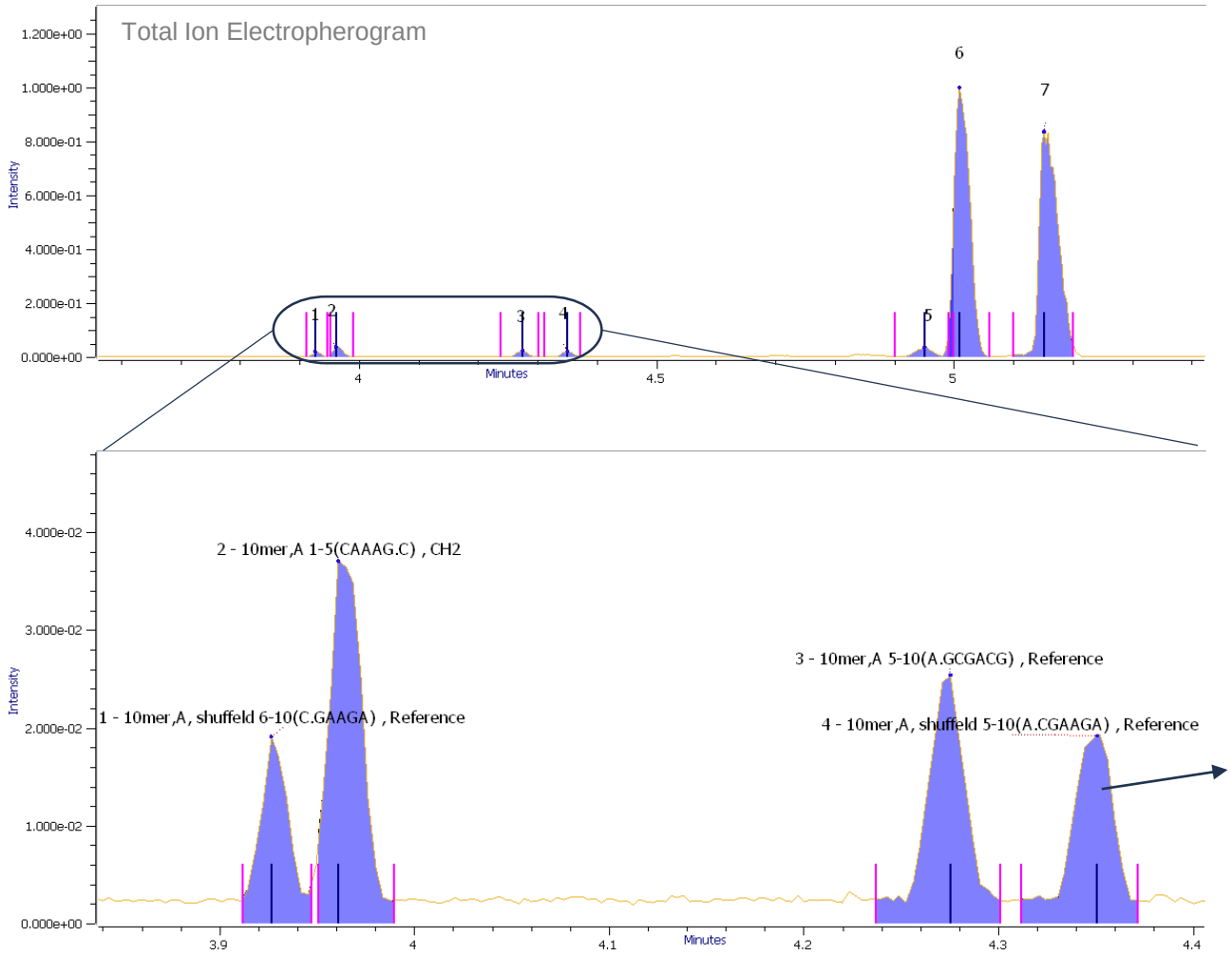
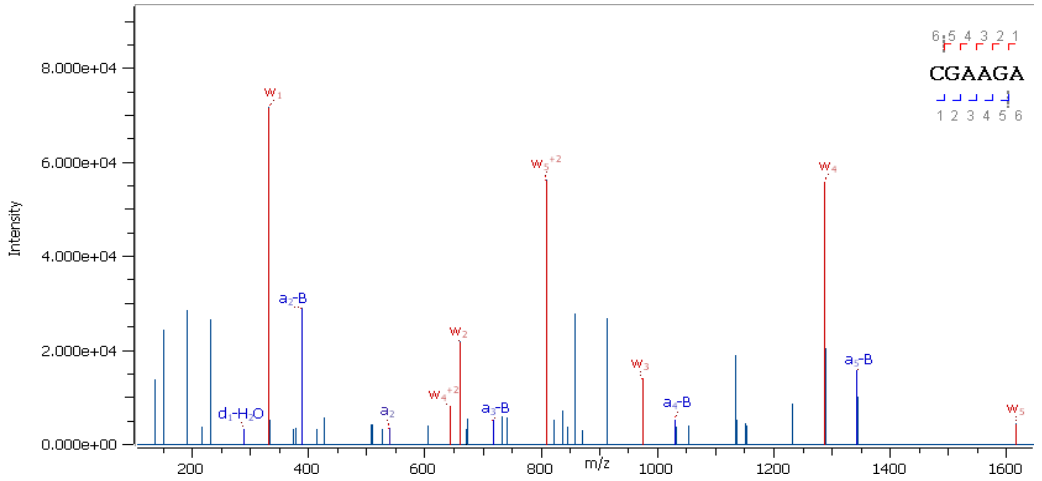
Identification and Quantification of Impurities



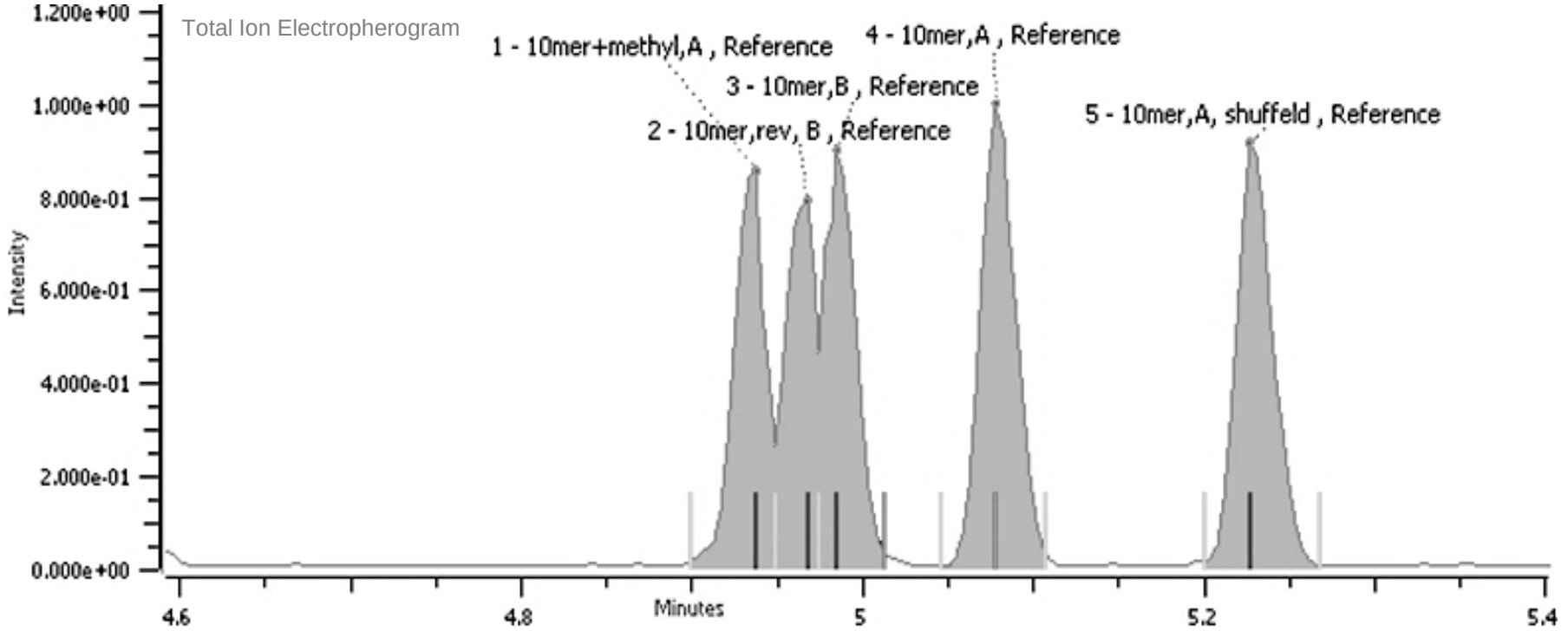
- Impurities present due to low purification grade
- Identification by MS/MS spectra
- Quantification based on MS level
- CE-MS allows for identification and quantification of impurities



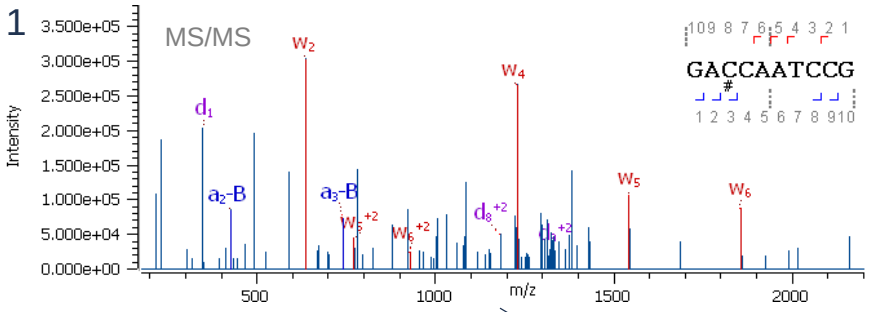
CE-MS Analysis of Semi-Complex Mixtures

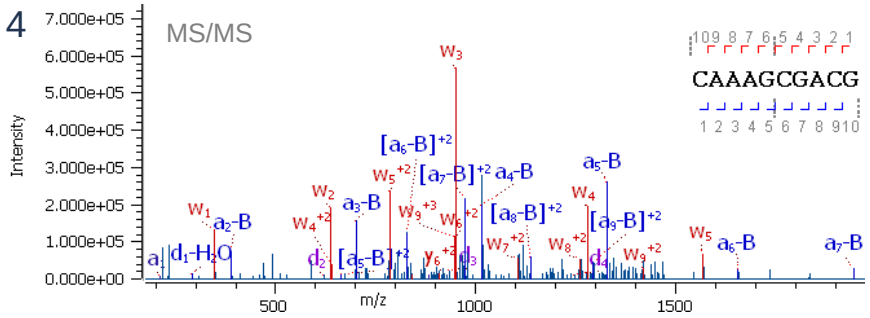
- Five oligonucleotides of same length and similar mass were analyzed
- A good separation was achieved
- Isobaric compounds can be separated

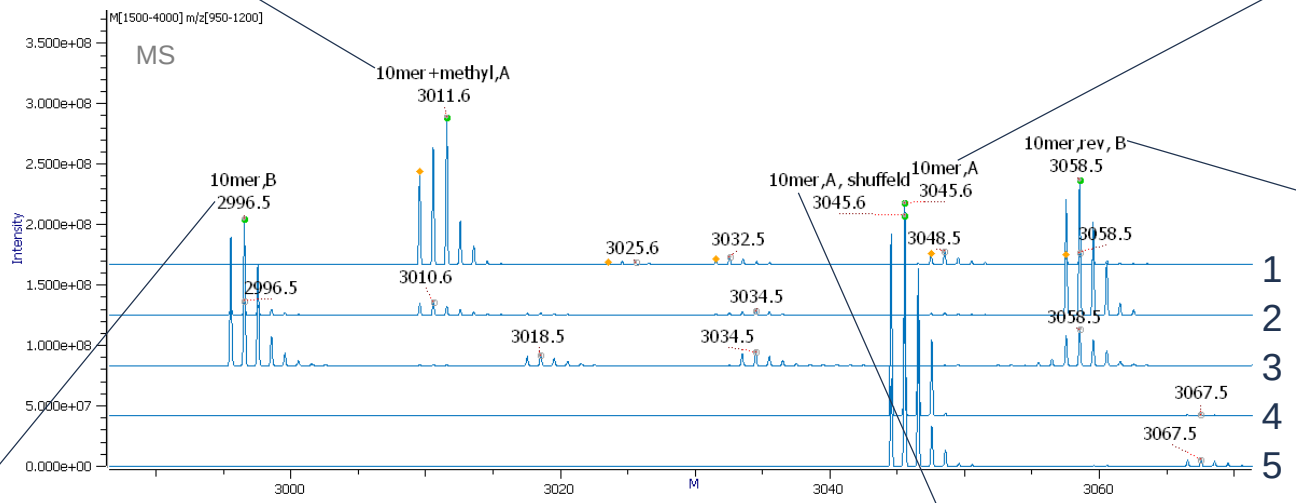
Name	Sequence	Molecular Weight [Da]	Peak
10mer, A	CAAAGCGACG	3,046	4
10mer, A shuffled	GCCACGAAGA	3,046	5
10mer+methyl, A	GAC [5MedC] AATCCG	3,012	1
10mer, B	GATCCAGACC	2,997	3
10mer,rev. comp., B	GGTCTGGATC	3,059	2

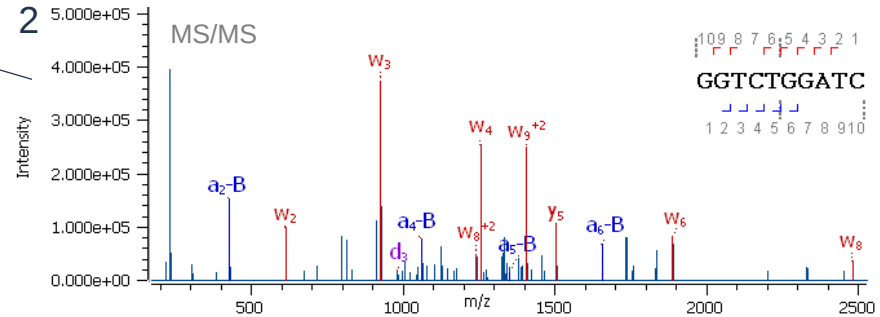


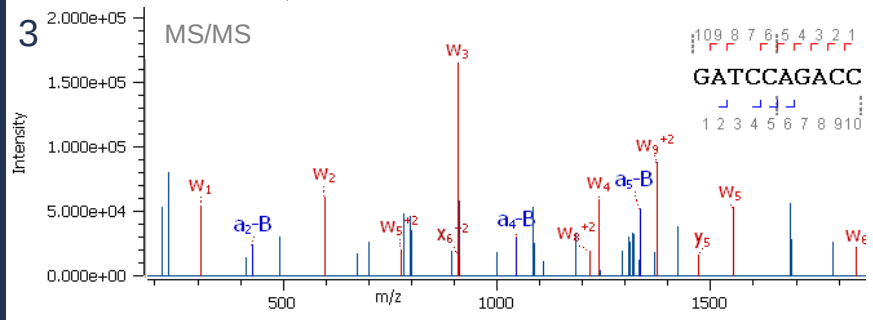
Sequence Confirmation by MS/MS

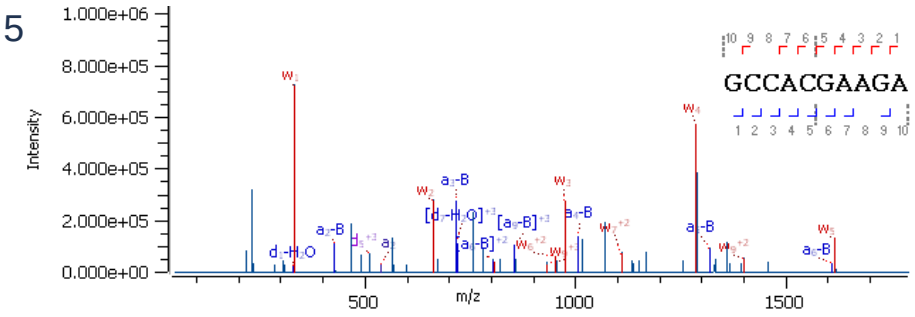












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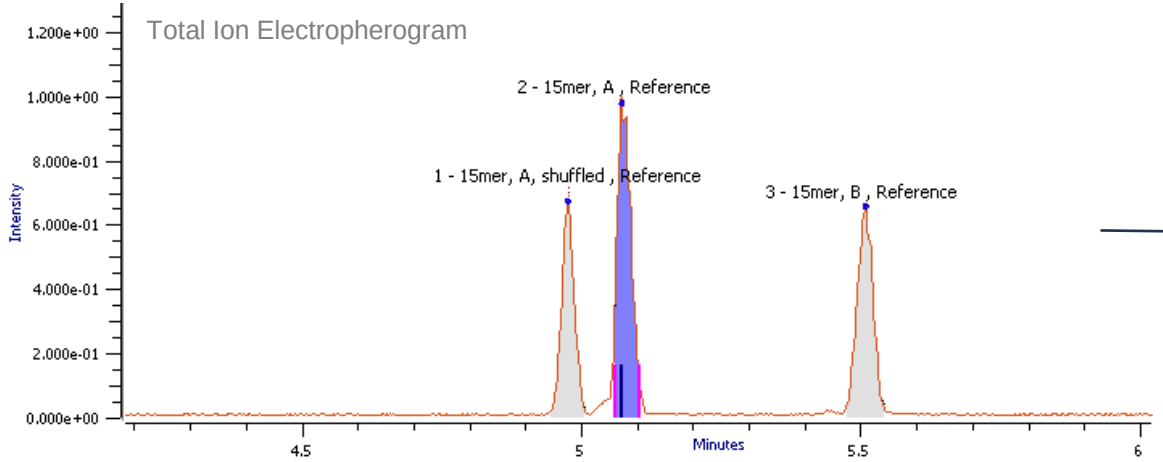
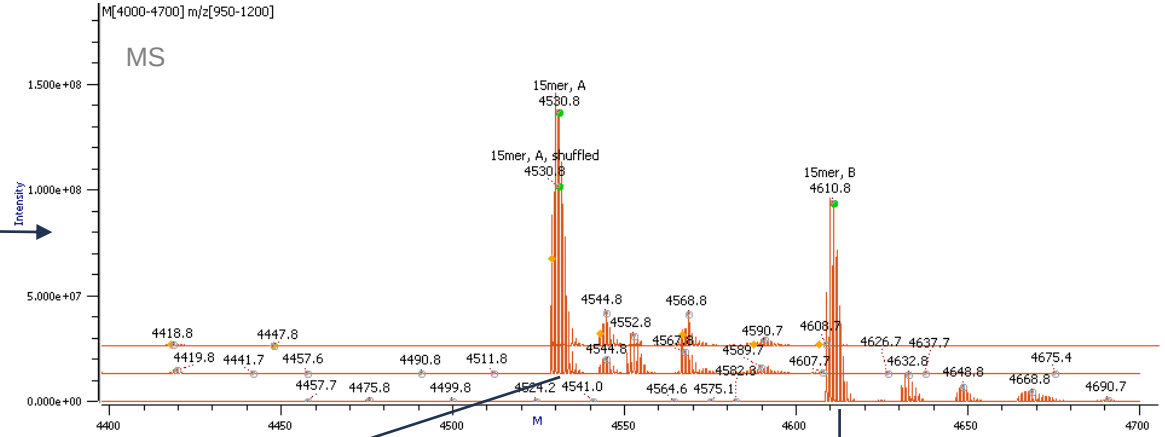
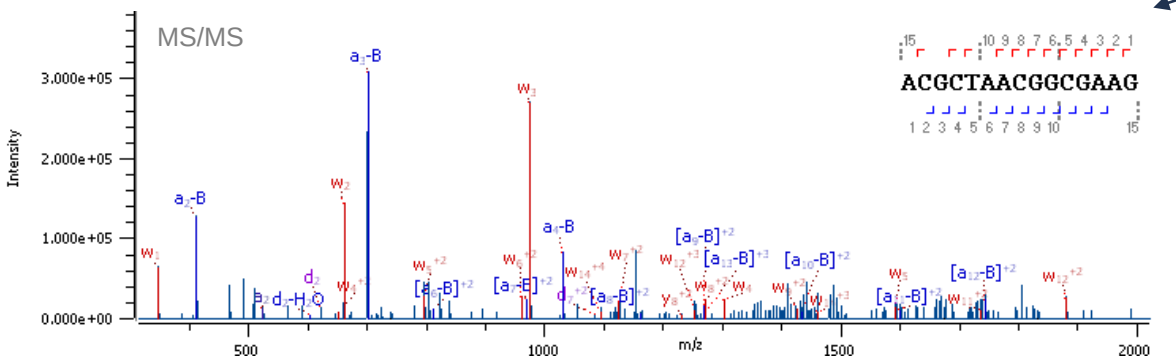
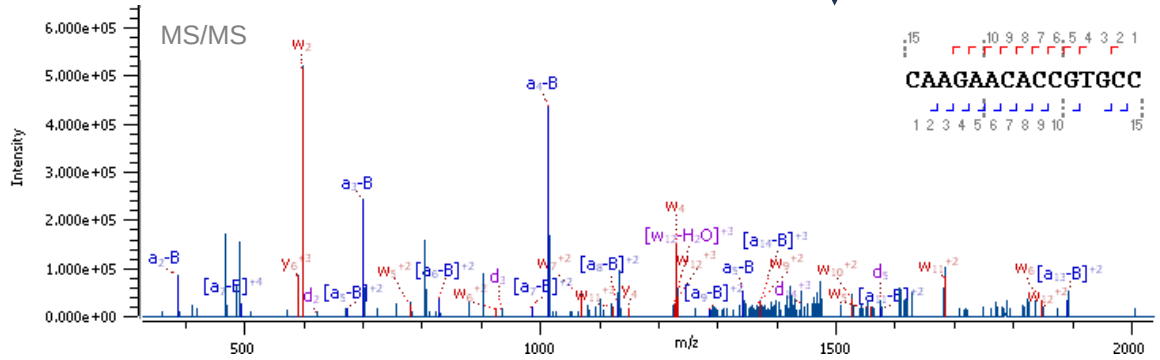
Sequencing of Longer Oligonucleotides









Conclusions

- CE-MS is a suitable tool for rapid analysis of oligonucleotides in quality control:
  - CZE separation using a ZipChip® device
  - Fast analysis (<10 min)
  - Low sample amounts needed
  - No ion-pairing agents required
  - Isobaric compounds can be separated
  - High-resolution accurate mass leads to high specificity
  - The quantification is linear over several orders of magnitude
  - High sensitivity (LOQ in the low nM range)

- Identification is easily achievable with BYOS® Oligos:
  - Molecular weight verification with accurate mass
  - Sequence verification via high quality MS/MS spectra
  - Fast and easy impurity analysis including identification and quantification

Acknowledgements

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908devices

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PROTEIN METRICS

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ProtaGene

Thank You

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