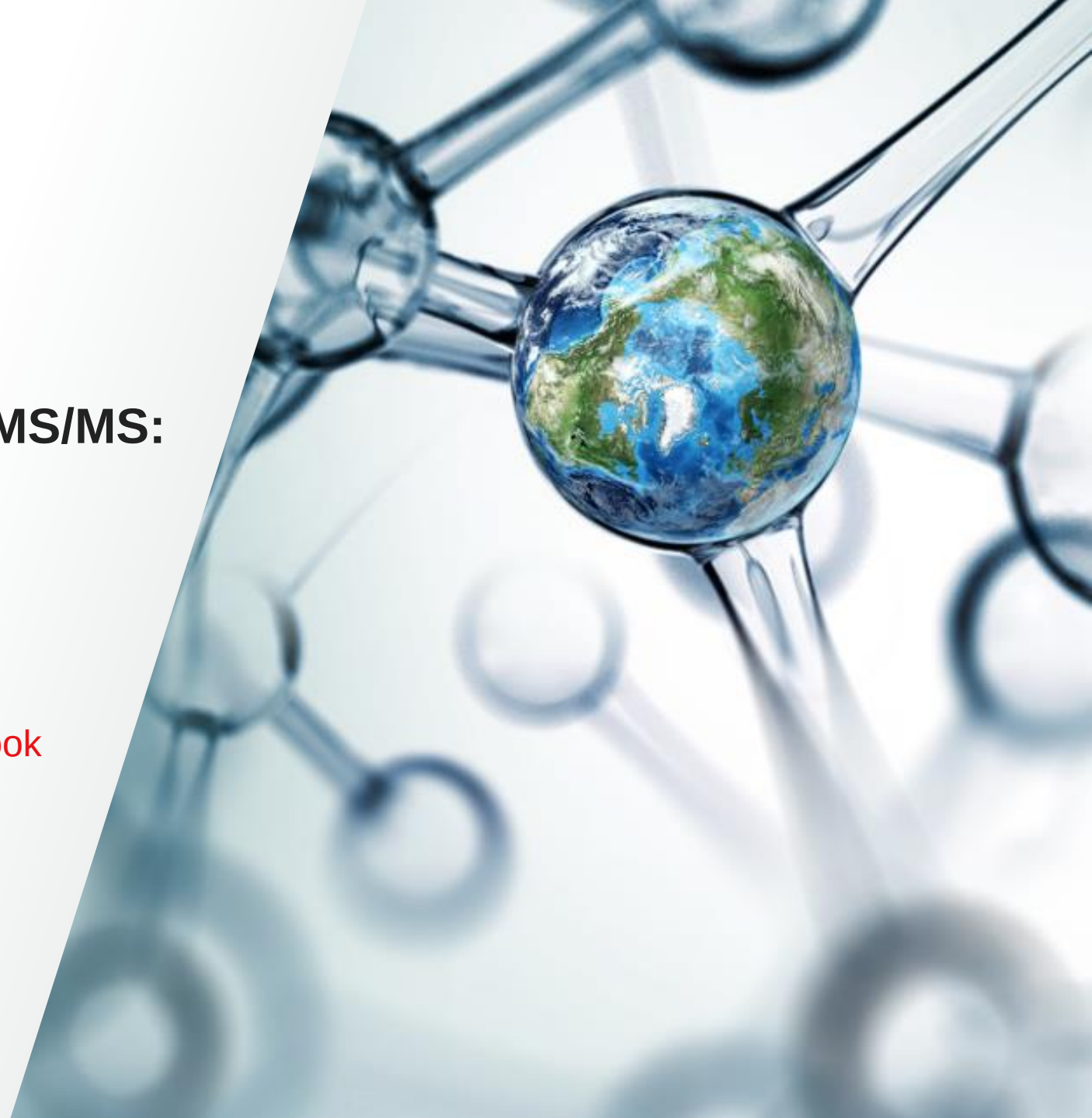
Oligonucleotide sequencing by LC-MS/MS:

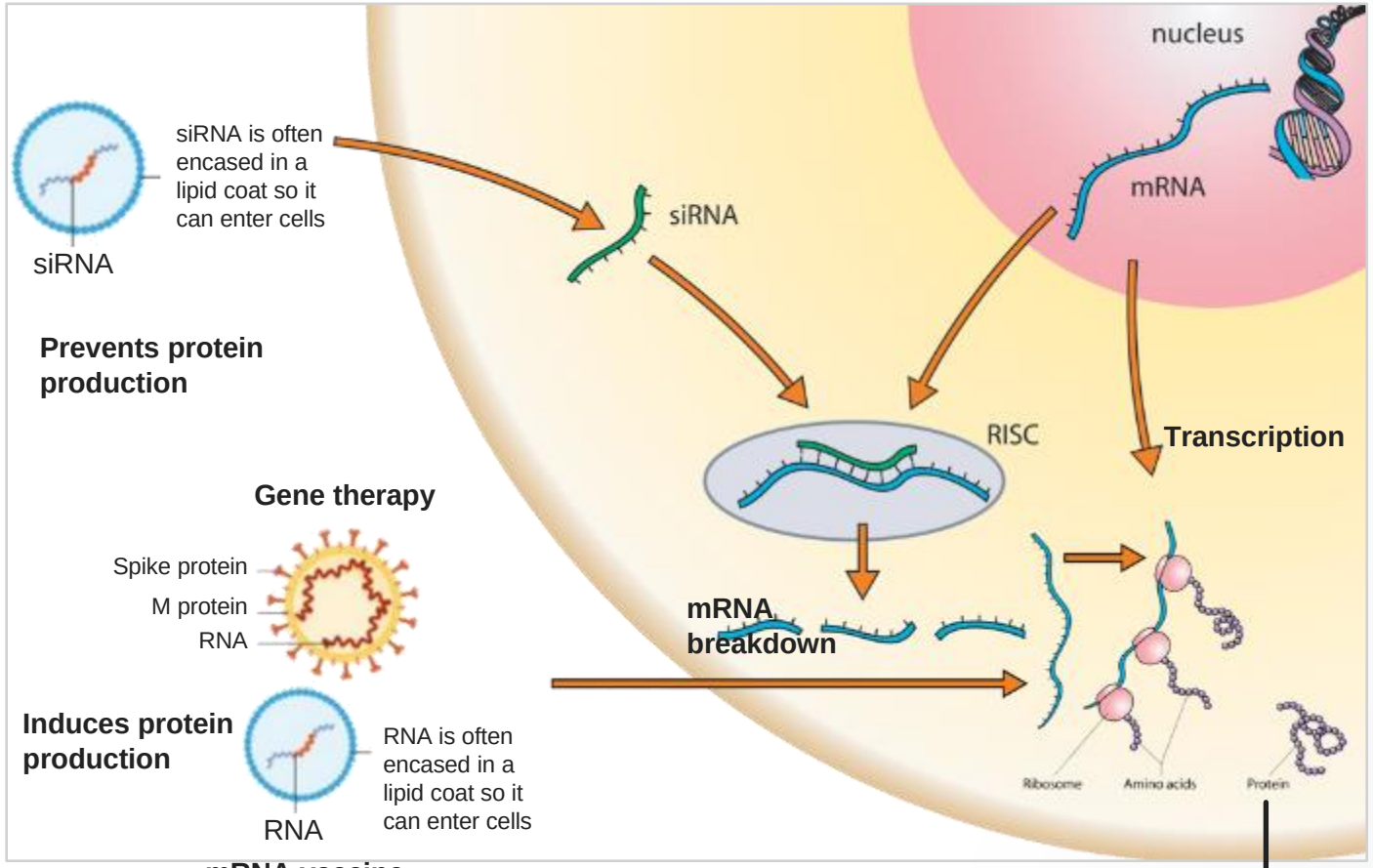
A novel Approach for Characterization and Quality Control of mRNA-based Vaccines and Biotherapeutics

Alexander Schwahn, Angela Criscuolo, and Ken Cook



The world leader in serving science

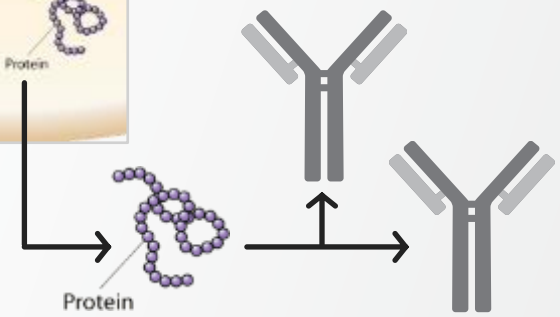
Why have oligonucleotides become so popular?

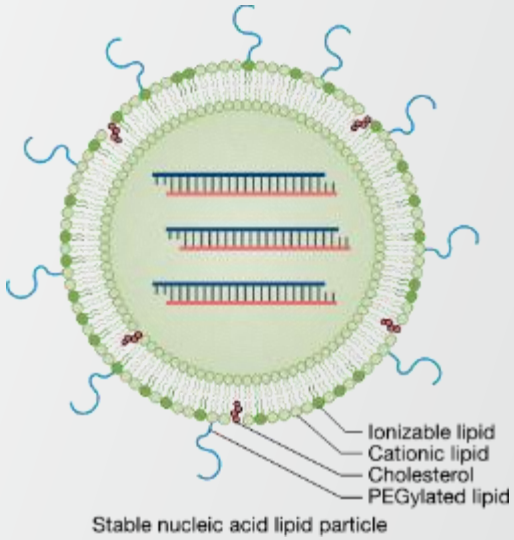


siRNA – short interfering RNA
Complimentary RNA strand to bind to specific mRNA and prevent protein production

Gene Therapy – replacement of a defective gene

mRNA Vaccine – adds mRNA into the cell to produce a viral coat protein





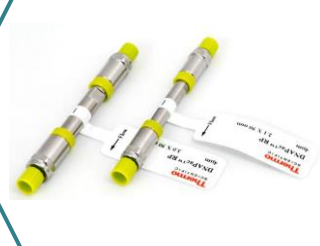
- LCMS – Oligonucleotide + Viral protein
- LCMS or CAD for lipid nano particle

mRNA sequencing

Our hardware and software for oligonucleotide analysis

Thermo Scientific™ SMART Digest™ RNaseT1 magnetic beads

Thermo Scientific™ DNAPac™ RP UHPLC columns



Thermo Scientific™ Vanquish™ UHPLC System

Thermo Scientific™ Orbitrap Exploris™ Mass Spectrometer

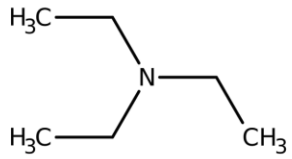
Thermo Scientific™ BioPharma Finder™ 5.1 and Chromeleon™ 7.3 Softwares

Methods benefit from our state-of-the-art Sample preparation, UHPLC, columns, Orbitrap technology, and software

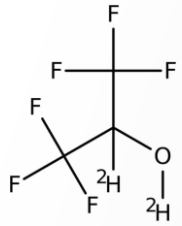
Reagent and consumable considerations

- Cleaning of UHPLC: Flush overnight to over the weekend with 100 mM Methanesulfonic acid
- Cleaning of MS: Clean transfer capillary with 0.1-1% Formic acid

UHPLC-MS grade



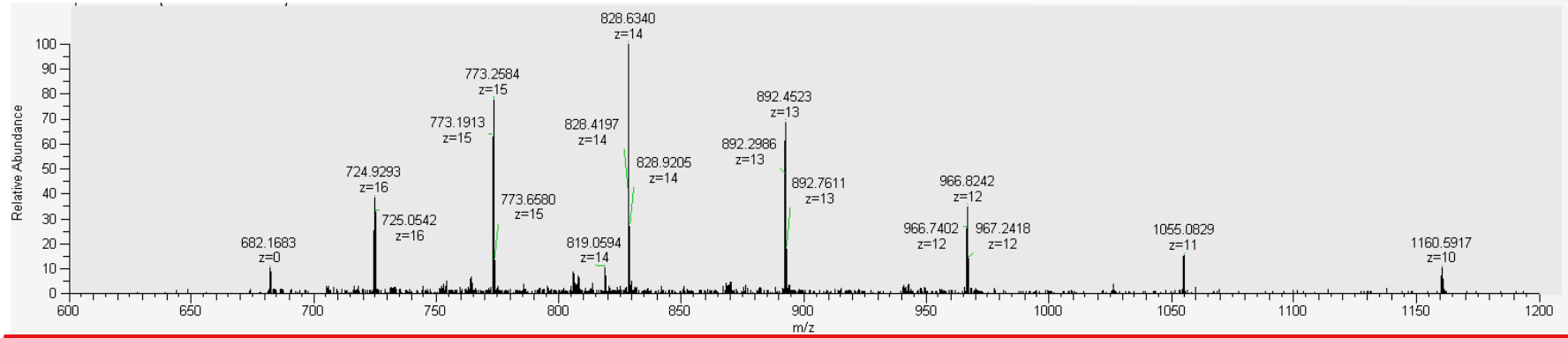
Thermo Scientific™ Triethylamine, 99.7% (PN 219510500)



Thermo Scientific™ Hexafluoro-2-propanol 99.9% (PN AC293410500)

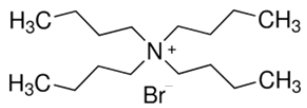
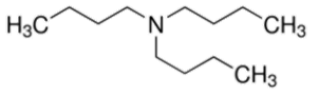
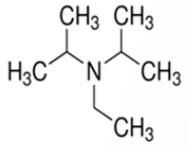
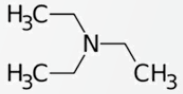
Use of plastic vials, glass absorbs metal ions and will cause adducts (PN C4000-11)

Thermo Scientific™ Dionex™ Methanesulfonic Acid Cation Eluent Concentrate for cleaning UHPLC (PN 080388)



UHPLC-MS grade solvents and additives to obtain clean spectra and increased sensitivity

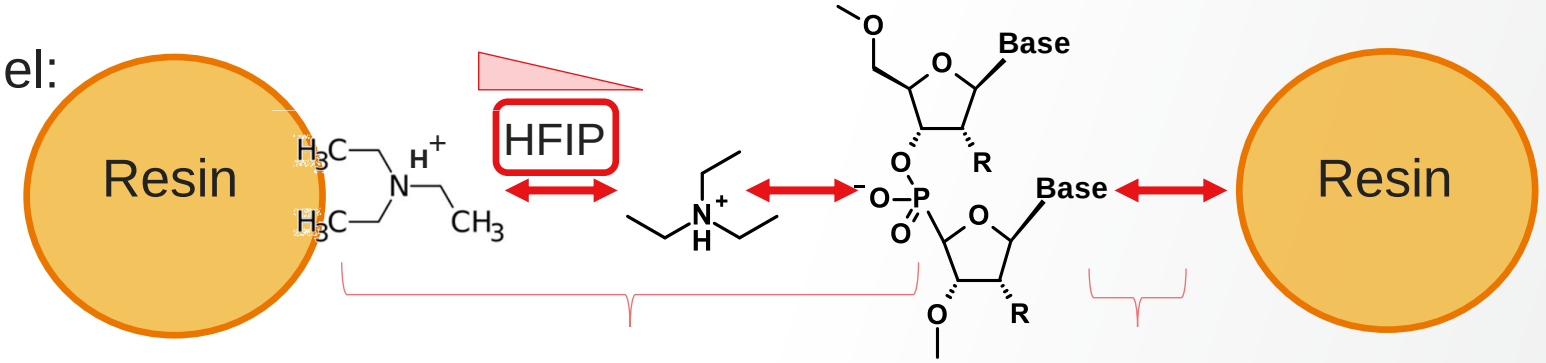
Amine Ion Pairs available for oligonucleotide separation

	Tetra-butylammonium bromide	Tributylamine	Di-isopropyl-ethylamine	Hexylamine	Triethylamine
					
Molar mass:	322.37 g/mol	180.39 g/mol	129.25 g/mol	101.19 g/mol	101.19 g/mol
Boiling point:	n/a (melting 103°C) degrades at 133°	214°C	127°C	131 °C	89°C

Decreasing hydrophobicity of ion-pair

Increasing sequence specificity separation

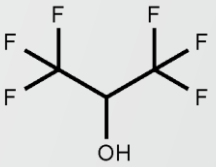
model:



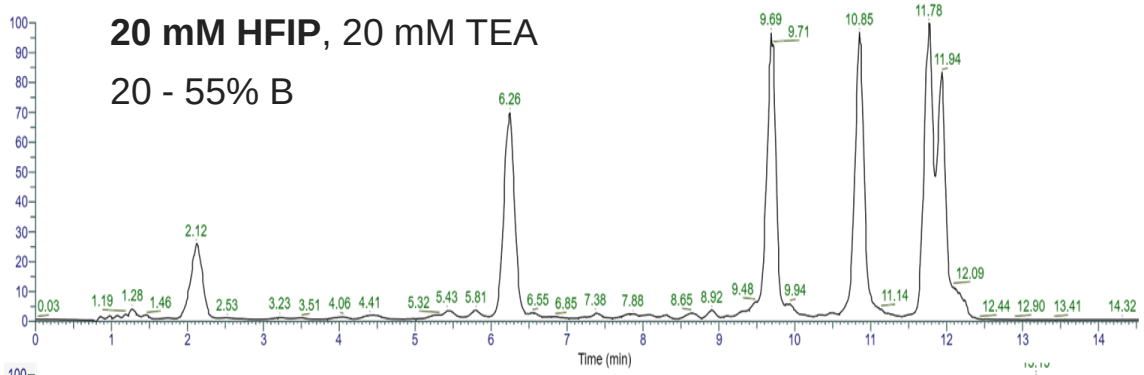
IP Interaction / pseudo-IEX with phosphate backbone

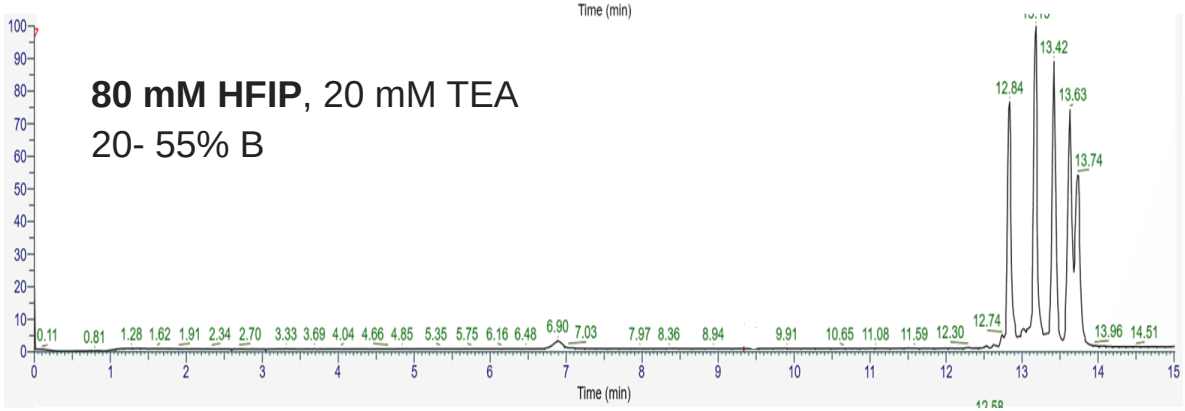
RP interaction with bases

Boiling point 58.2 °C, **HFIP** will evaporate first and change the ratio of IP to acid modifier



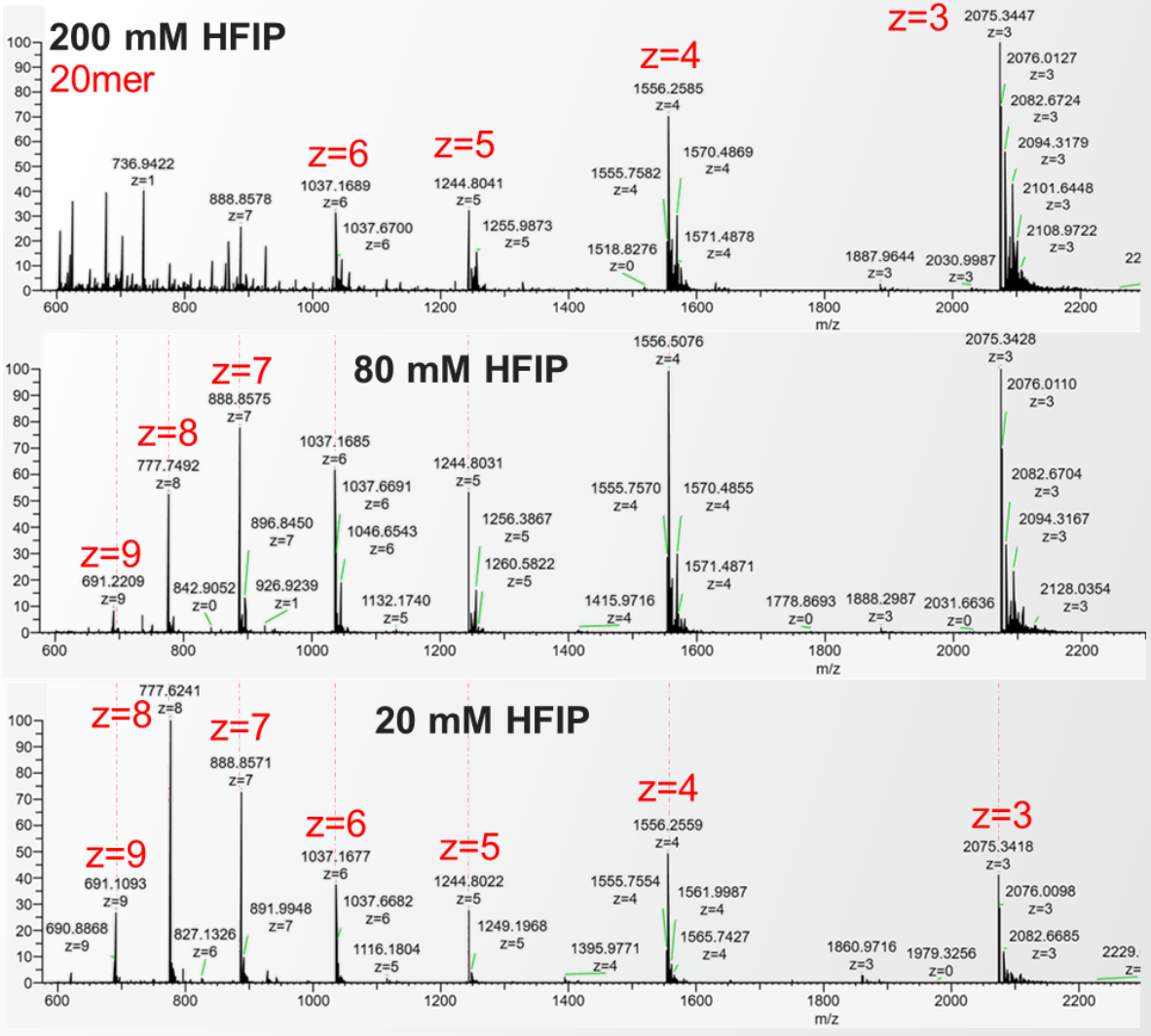
HFIP increases retention and reduces chart state distribution

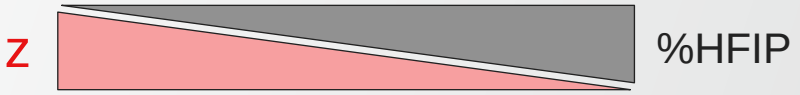




Eluent A = 20 mM TEA in H_2O
Eluent B = 20 mM TEA with 25% methanol
HFIP concentration varied between 20 and 80 mM

The charge state distribution effects sequencing efficiency





Oligonucleotide characterization

MS settings on Thermo Scientific™ Orbitrap Exploris™ systems

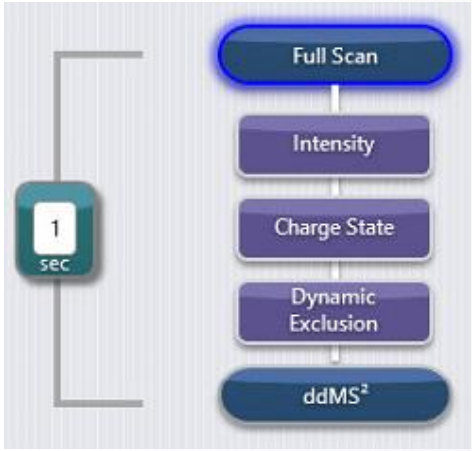
Full Scan for <40mer

- Orbitrap Resolution: 120,000
- Polarity: Negative

Full Scan for >40mer (e.g., 100mer)

- Intact Protein Mode (Low Pressure)
- Orbitrap Resolution: 240,000
- Polarity: Negative
- Microscans: 2

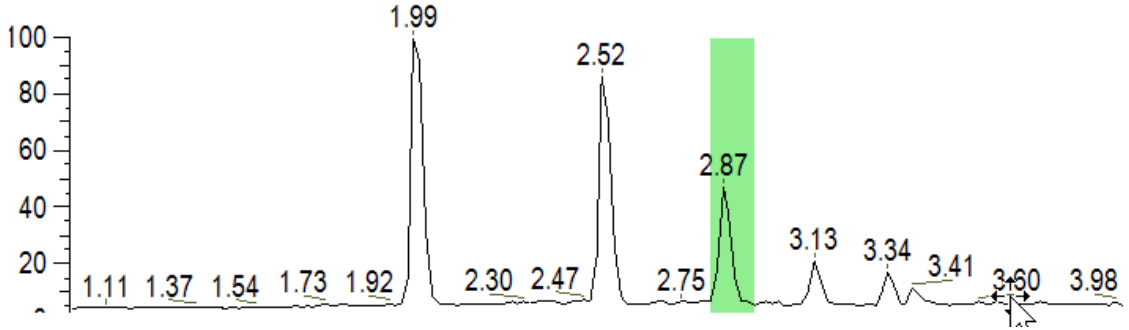
ddMS²

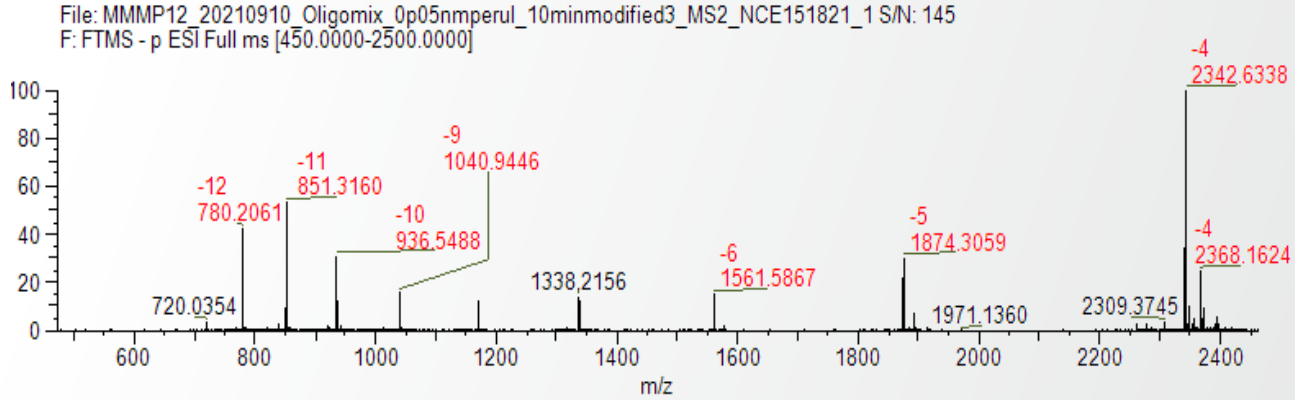


- Peptide Mode
- Orbitrap Resolution (at m/z 200): 60,000 or 120,000 (MS1), 30,000 (MS2)
- Polarity: Negative
- **Stepped Normalized Collision Energy (NCE):** 10-12-14 to 20-22-24 (18-20-22 for impurity analysis)

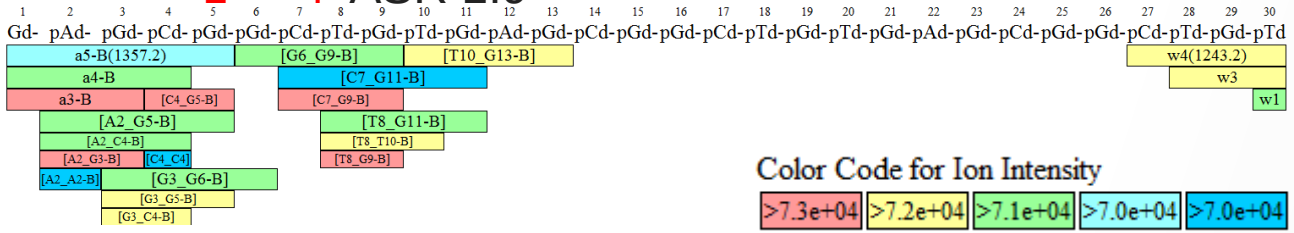
- Very little adduct formation
- 120,000 resolution with high sensitivity
- Good sensitivity of larger fragments
- Good MS/MS data with fragments up to 60nt

Stepped collision energy optimization - 30mer



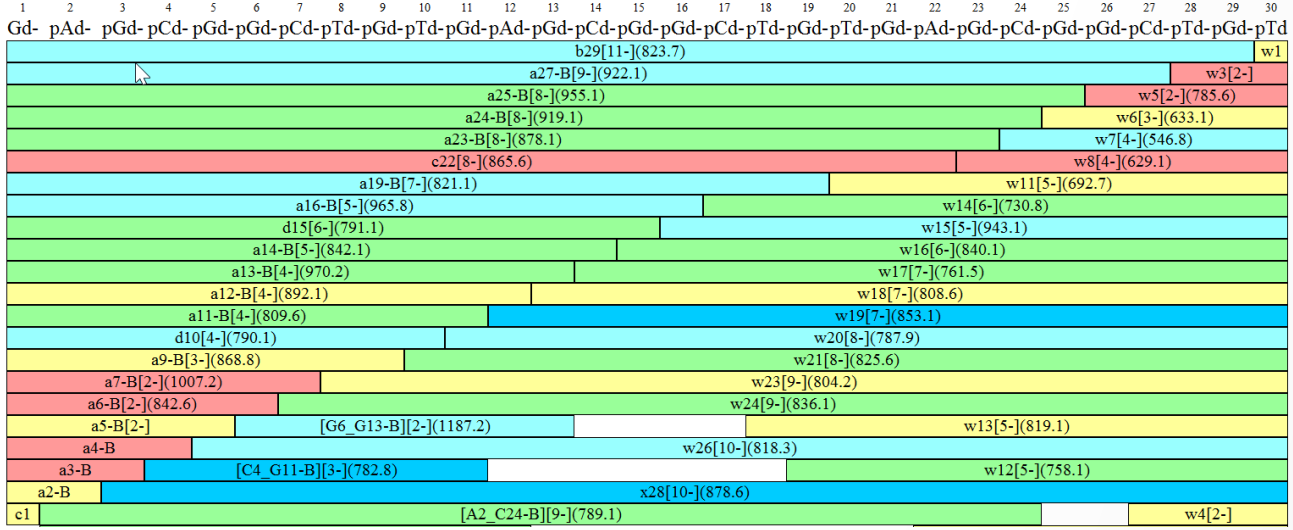


z = -4 ASR 1.9



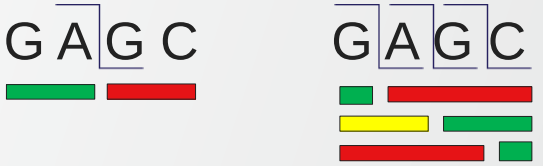
Color Code for Ion Intensity

z = 11 ASR = 1.0



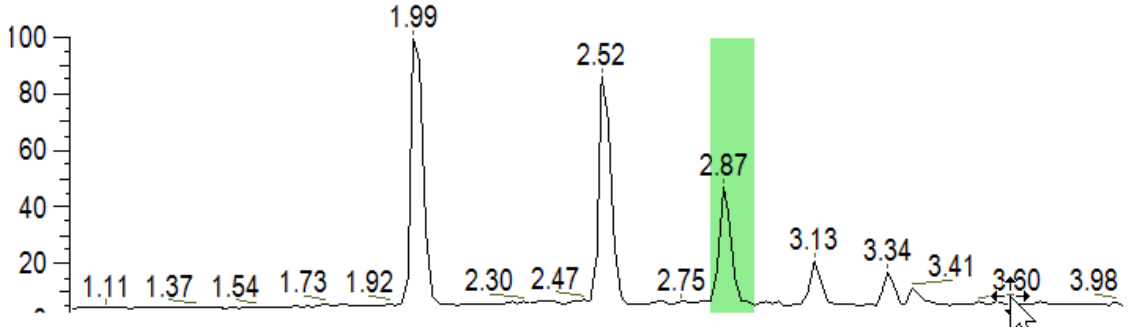
Average Structural Resolution (ASR):

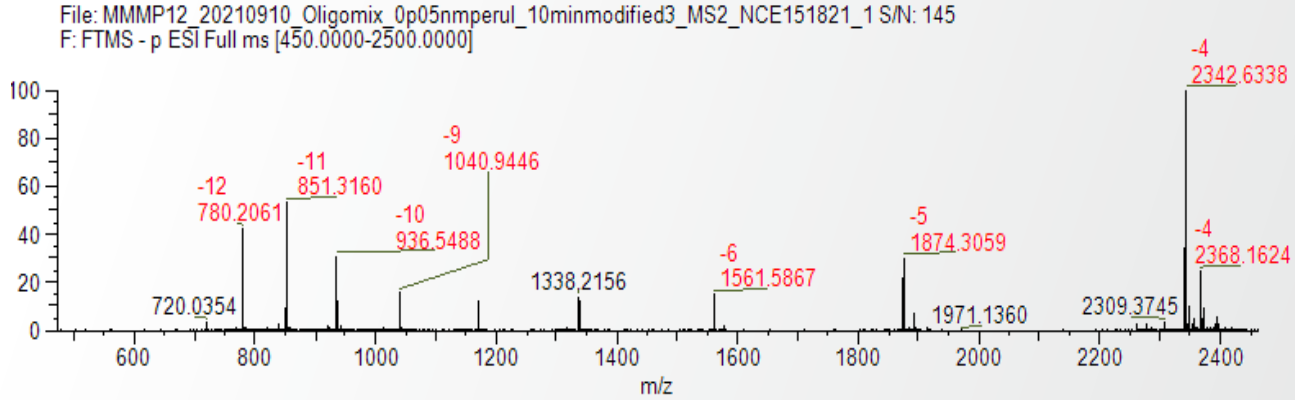
$$ASR = \frac{\text{Number of Nucleotides (n)}}{\text{Number of Bonds Found (b) + 1}}$$



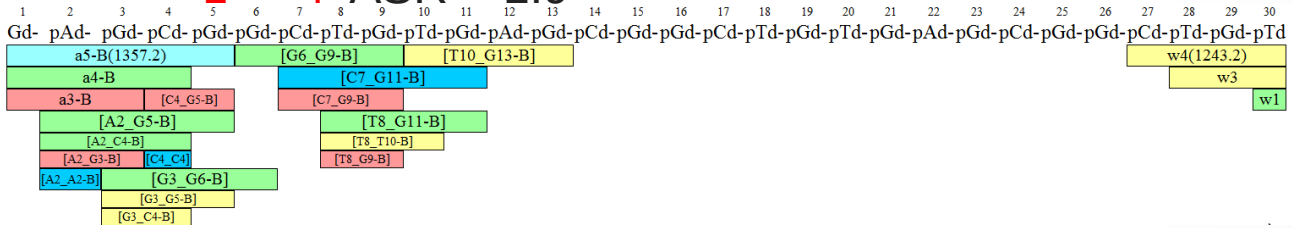
n	4	4
b	1	3
ASR	**2**	**1**

Stepped collision energy optimization - 30mer

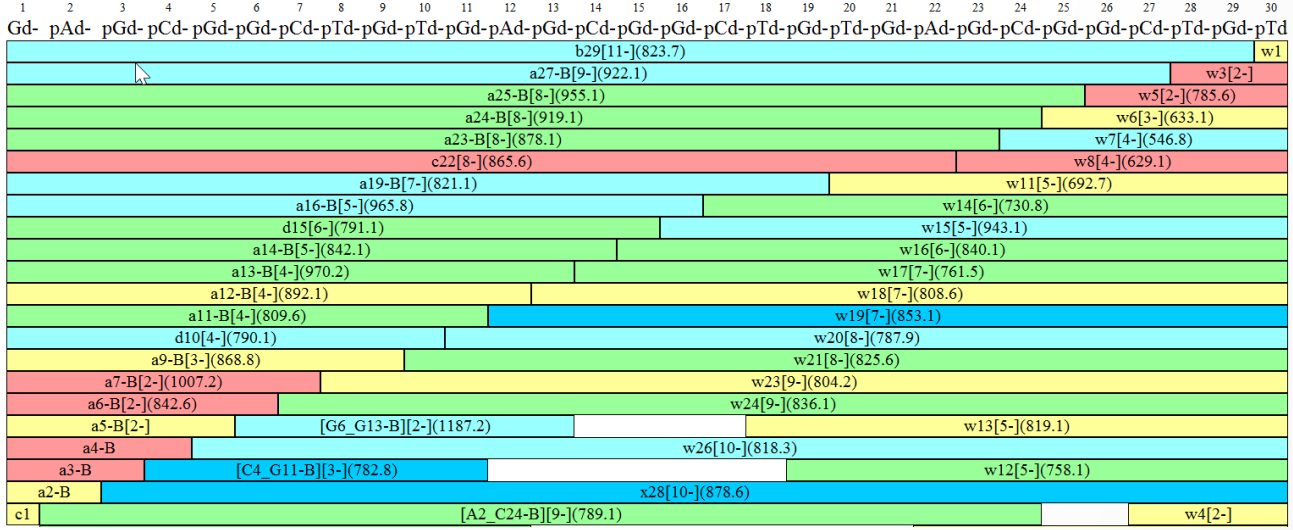




z = -4 ASR = 1.9



z = -11 ASR = 1.0

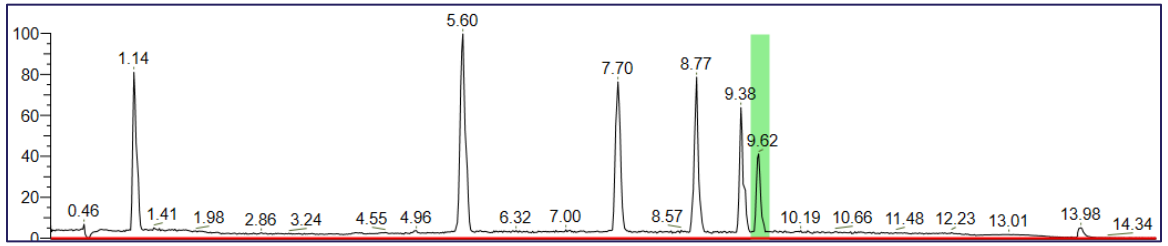


Lower stepped NCE* Higher stepped NCE

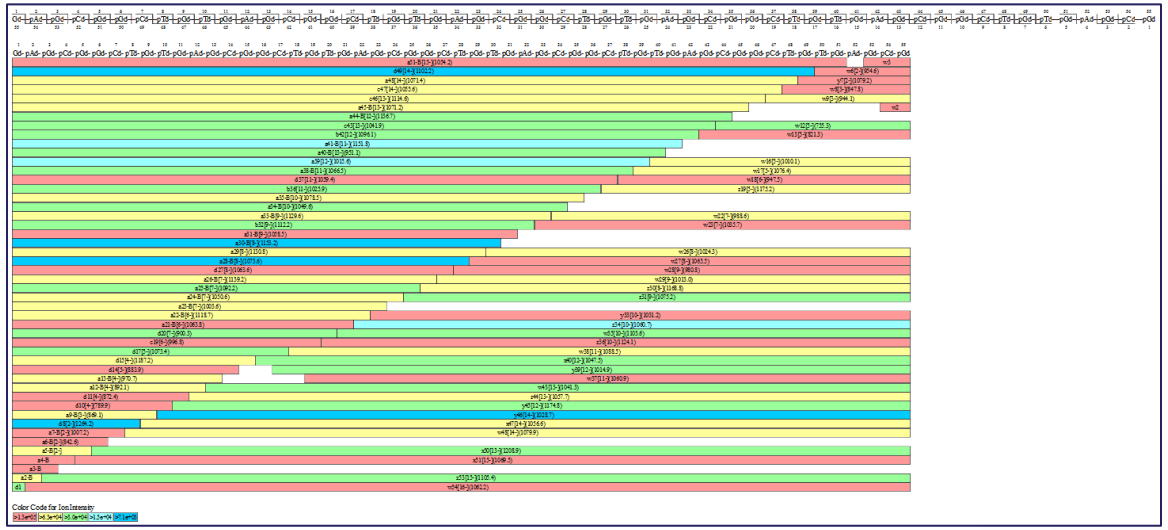
z	ASR	ASR
-12	1.0	1.4
-11	1.0	1.4
-10	1.1	1.5
-9	1.2	1.6
-7	1.1	1.8
-6	1.2	2.4
-5	1.6	2.2
-4	1.9	2.2

*NCE … normalized collision energy used for HCD fragmentation

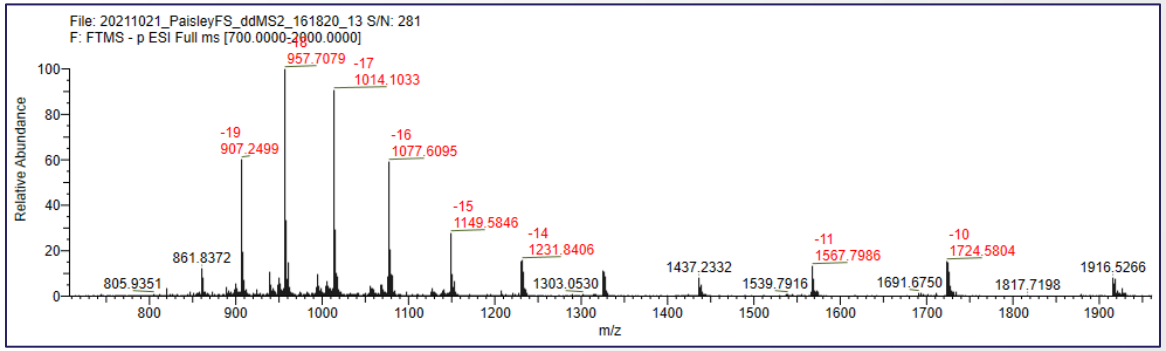
55mer, NCE 13/15/17 (15/18/21)



-16 ASR = 1.0



Confidence 99.3



z	NCE 13/15/17	(15/18/21)
-19	**ASR = 1.0**	(1.1)
-18	**ASR = 1.0**	(1.1)
-17	**ASR = 1.0**	(1.1)
-16	**ASR = 1.0**	(1.1)
-15	ASR = 1.0	(1.1)
-14	ASR = 1.1	(1.3)
-13	ASR = 1.3	(1.8)
-12	ASR = 1.6	

- Lower stepped collision energies (NCE) give better fragmentation, especially for longer oligomers
- Higher charge state ions result in better sequence coverage (ASR = 1)

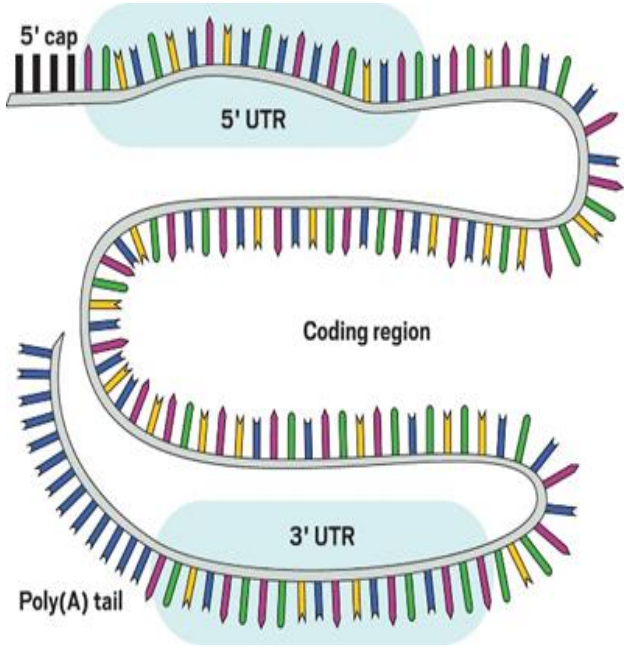
Provided values are without internal fragments

Limited digestion with single strand ribonucleases

Problem statements:

- RNase T1 complete cleavage gives fragments that are too short – requires a partial digest
- RNase T1 works very fast and is very difficult to control and stop effectively
- RNAseT1 in-solution digests usually contaminate the analytical column with nuclease
- Until now any mRNA fragmentation work is done using multiple Nuclease digestions, combining the data

Solution: - RNase T1 immobilised on magnetic beads



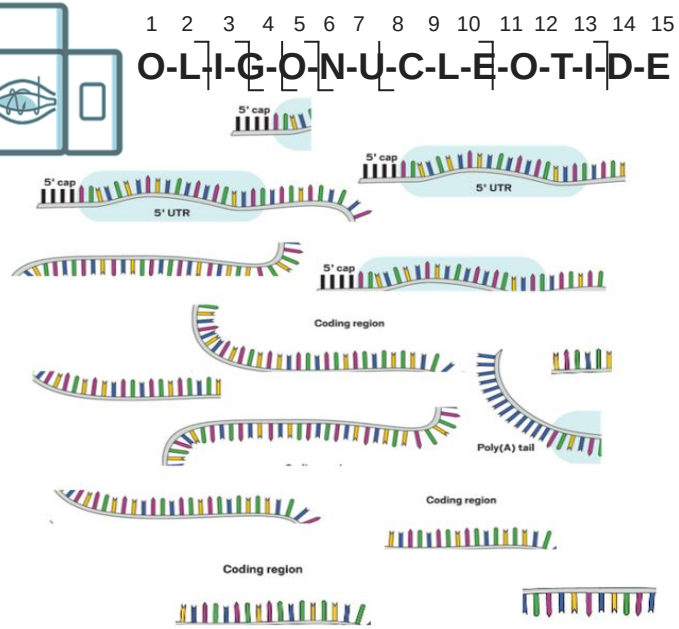
mRNA

Description	Part Number
RNase T1 Mag Bulk Kit	60120-101
RNase A Mag Bulk Kit	60120-102

Partial digestion

Introduce beads to start digestion

Remove beads to end digestion



LCMS/MS and data annotation

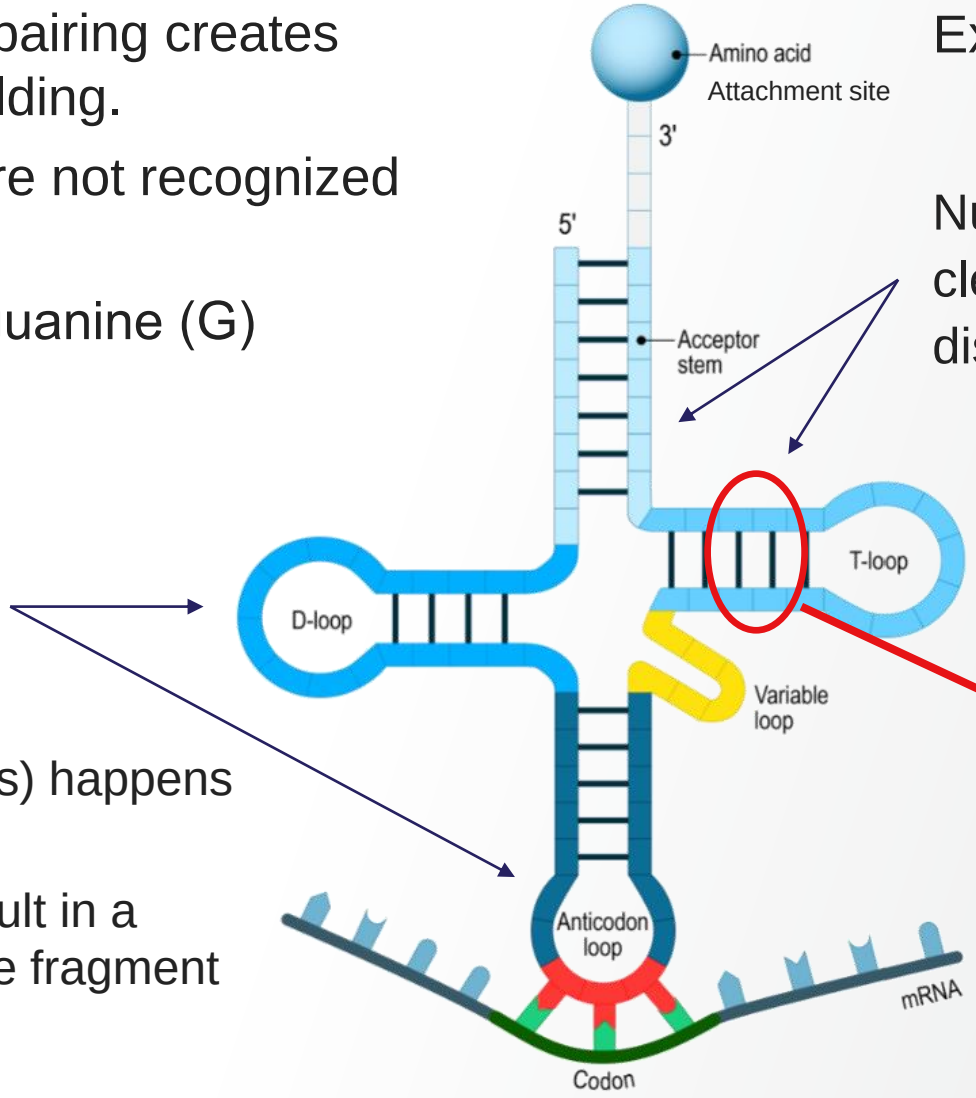
alexander.schwahn@thermofisher.com | ISC2022

Structure of RNA and digestion preferences

- RNA intra-molecular base-pairing creates secondary structure and folding.
- Double stranded regions are not recognized as substrate by RNAse T1.
- RNase T1 cleavage: 3’ of guanine (G)

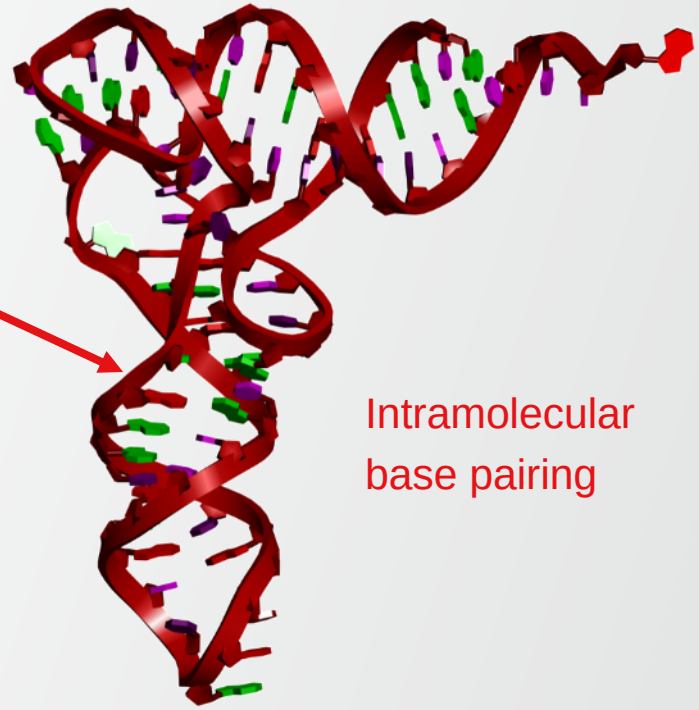
RNase T1 will cleave preferentially

- Complete digestion (of ss-sites) happens very quickly
- the mRNA-specific folding result in a characteristic and reproducible fragment pattern



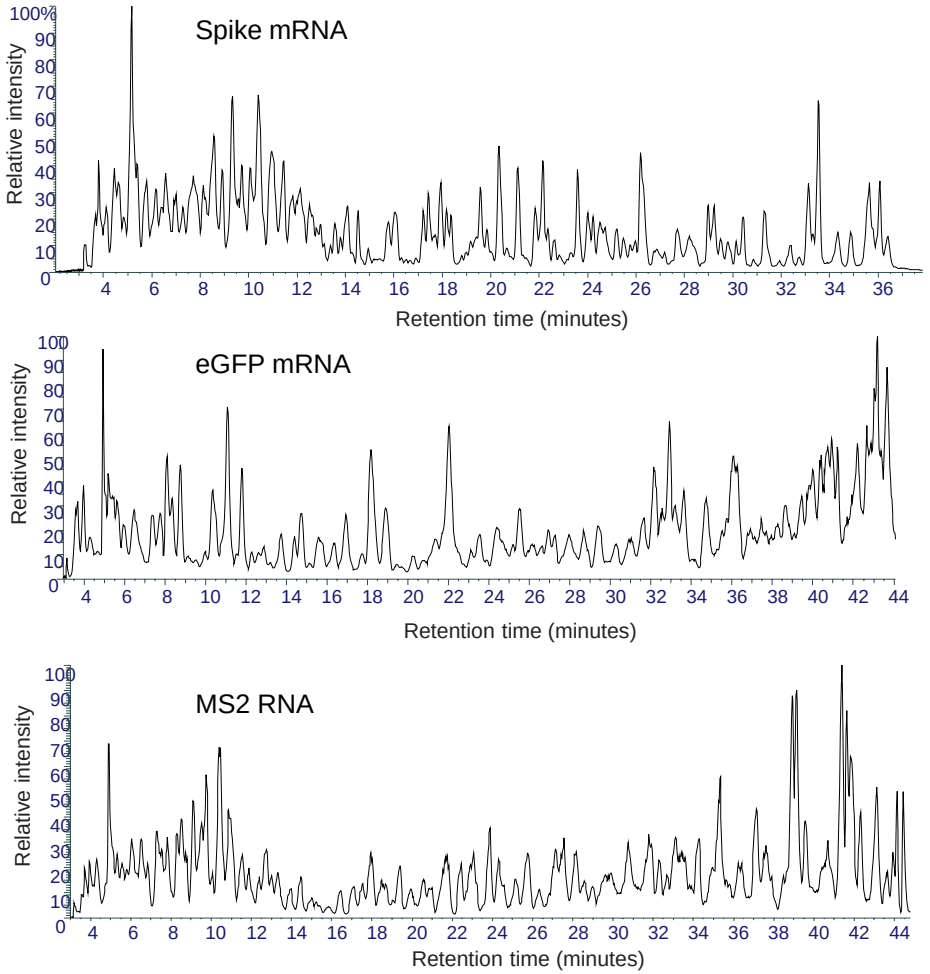
Example: tRNA

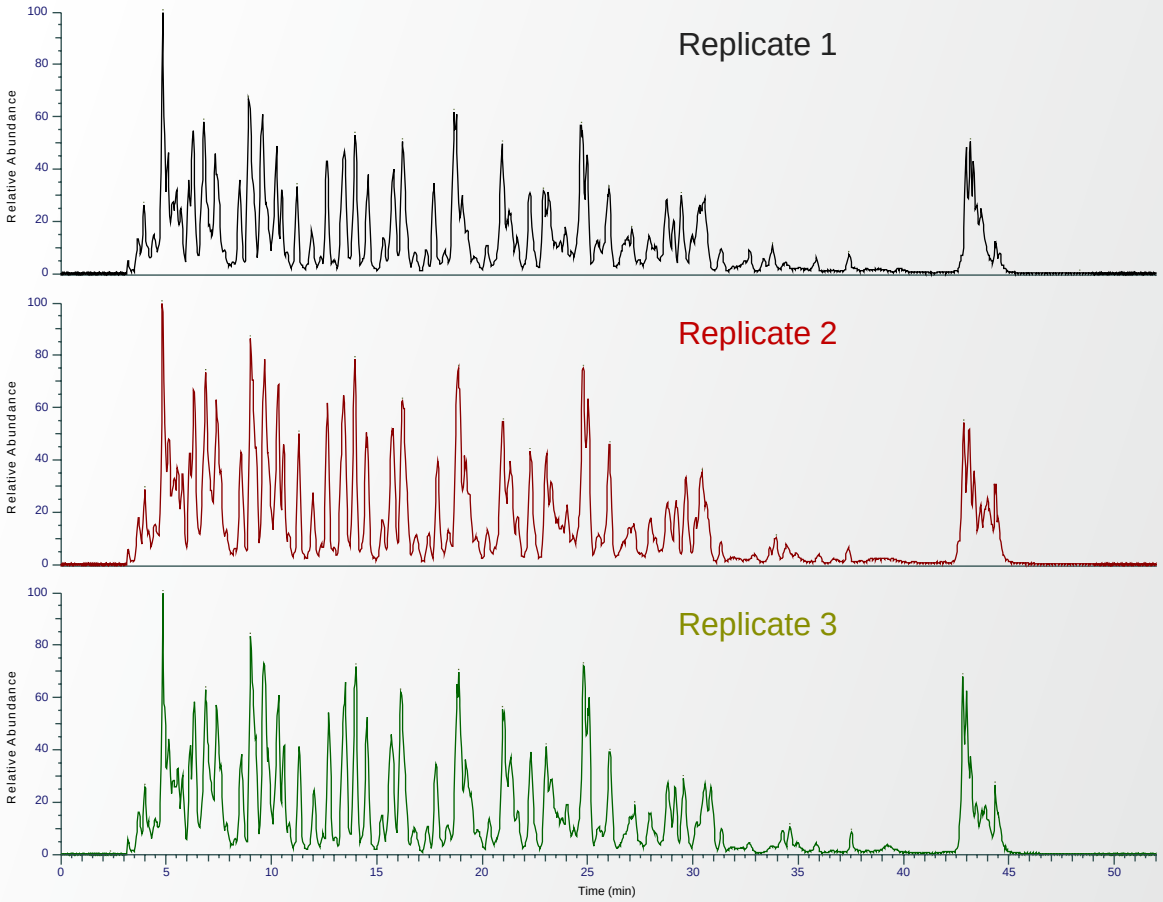
Nuclease T1 will not cleave until disrupted



Intramolecular base pairing

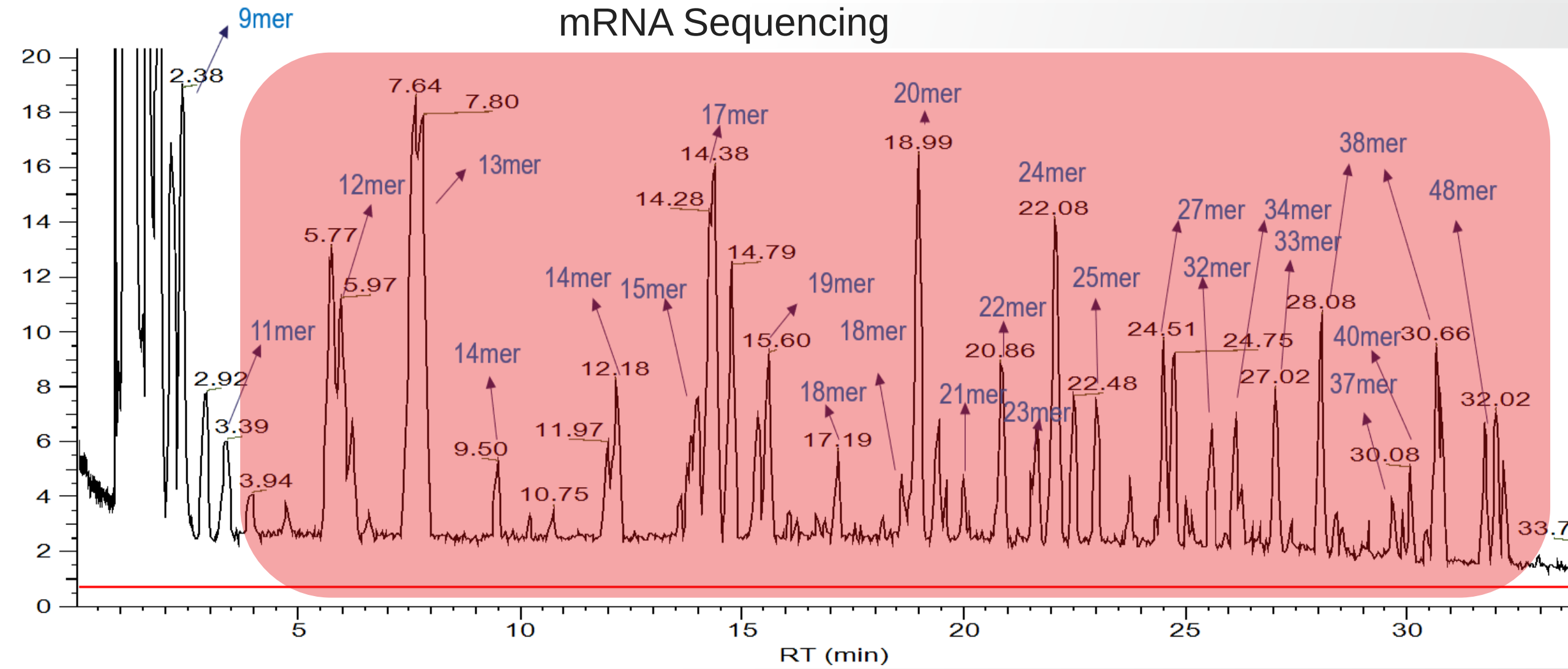
Reproducibility and selectivity





- This can be used as a QC test with UV only or MS
- The fragmentation pattern is specific for each mRNA
- The high reproducibility with a simple to use partial digest makes this possible as a QC test
- Using the partial digest also gives structural folding information

Why is it important to have good separation from 10-60mer?

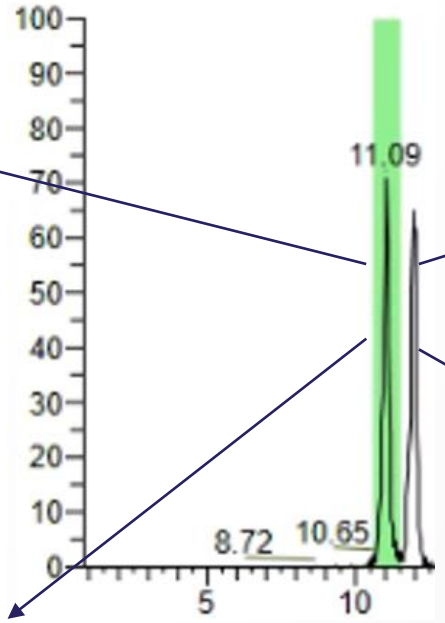


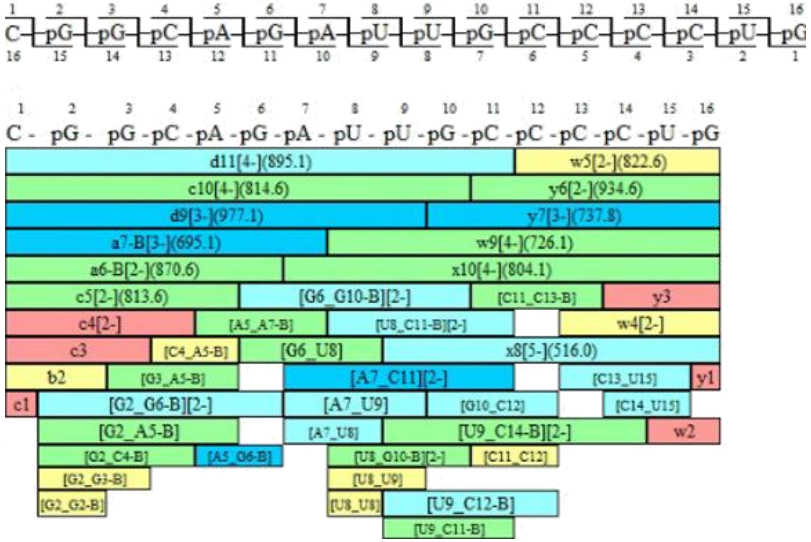
Identification and separation of sequence isomers

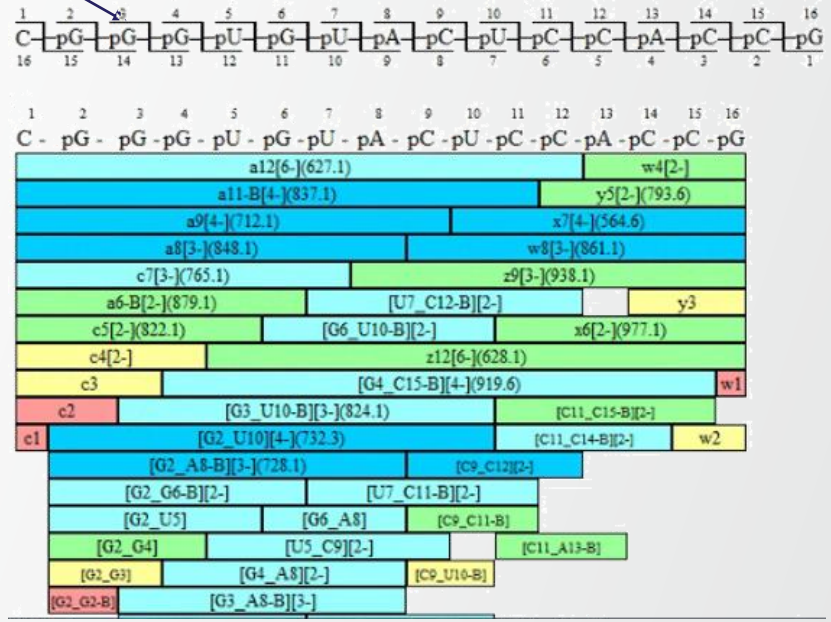
Theoretical monoisotopic mass 5131.6658 Da

5’CGGGUGUACUCCACCG$_{cP}$3’

5’-CGGCAGAUUGCCCCUG$_{cP}$-3’



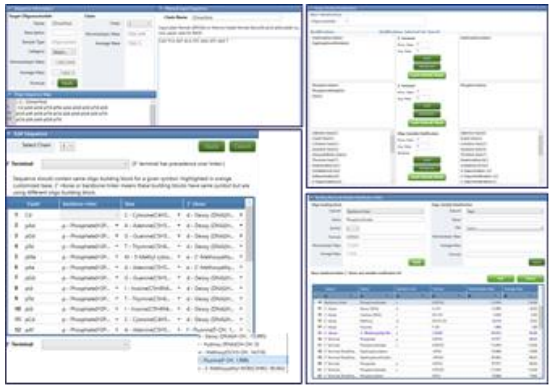


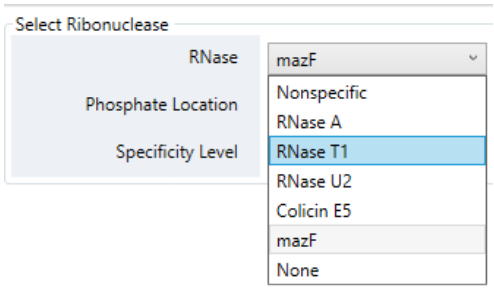


mRNA analysis - software

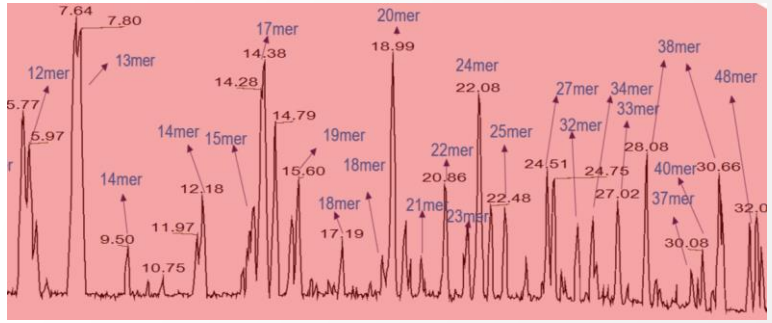
Expanding modalities for processing and supporting vaccine development

Sequence input → ribonuclease selection → mRNA component detection → identification confirmation

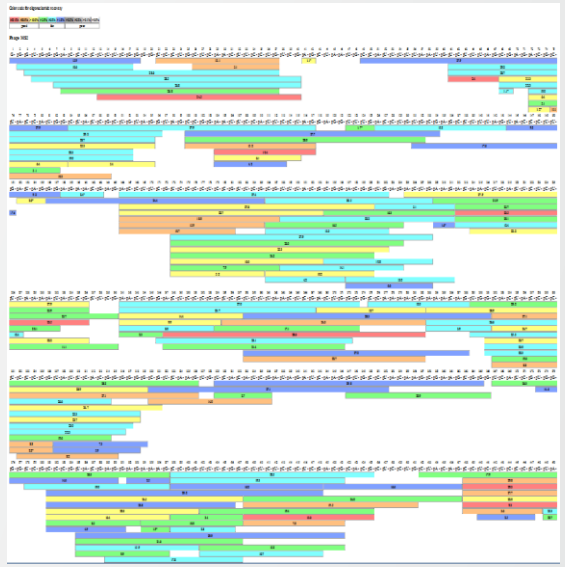




Ribonuclease selection includes common RNases



Chromatogram of digestion fragments from the mRNA sample.



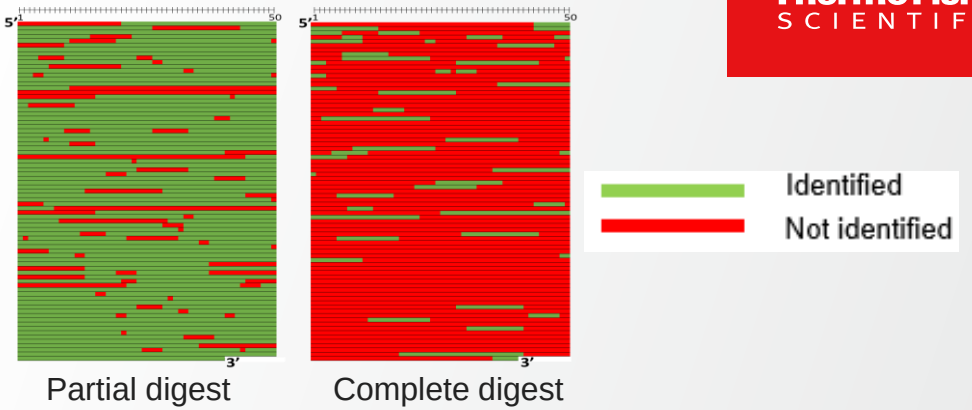
Sequence coverage map, automatic annotation and % coverage calculation

Spike protein mRNA sequencing data

Oligonucleotide	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:SPike	7011	66.8%	99.7%	100.00%

With filters to prevent false positives

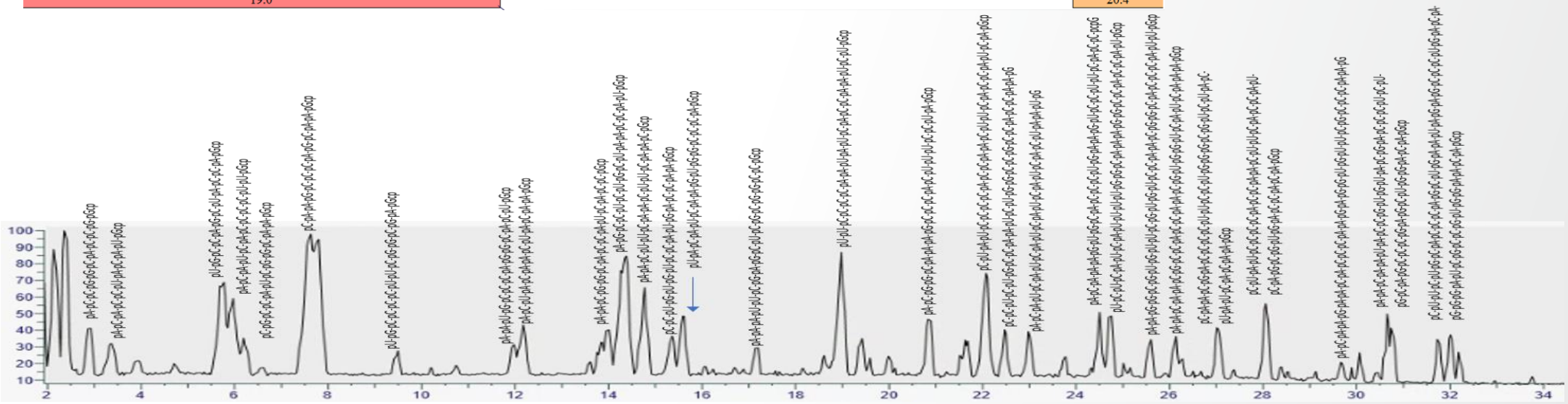
Oligonucleotide	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:SPike	3387	28.6%	89.3%	100.00%
1:SPike*	3387	28.6%	0.0%	100.00%
Unidentified	7755	71.4%		



Partial digest

Complete digest

- No wrong cleavages
- Reproducible fragment pattern
- Useful fragment sizes
- Structural information
- No false positives



Summary

- Optimization of fragmentation for sequencing linked to chromatography pH [charge selection]
- HCD fragmentation energies linked to the size of the oligonucleotide
- The eluents are stable
- We can pick up known modifications
- Routine sequencing of over 55nt long oligonucleotides with optimizes HCD fragmentation
- Around 60 minutes for the entire analysis including sample preparation

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