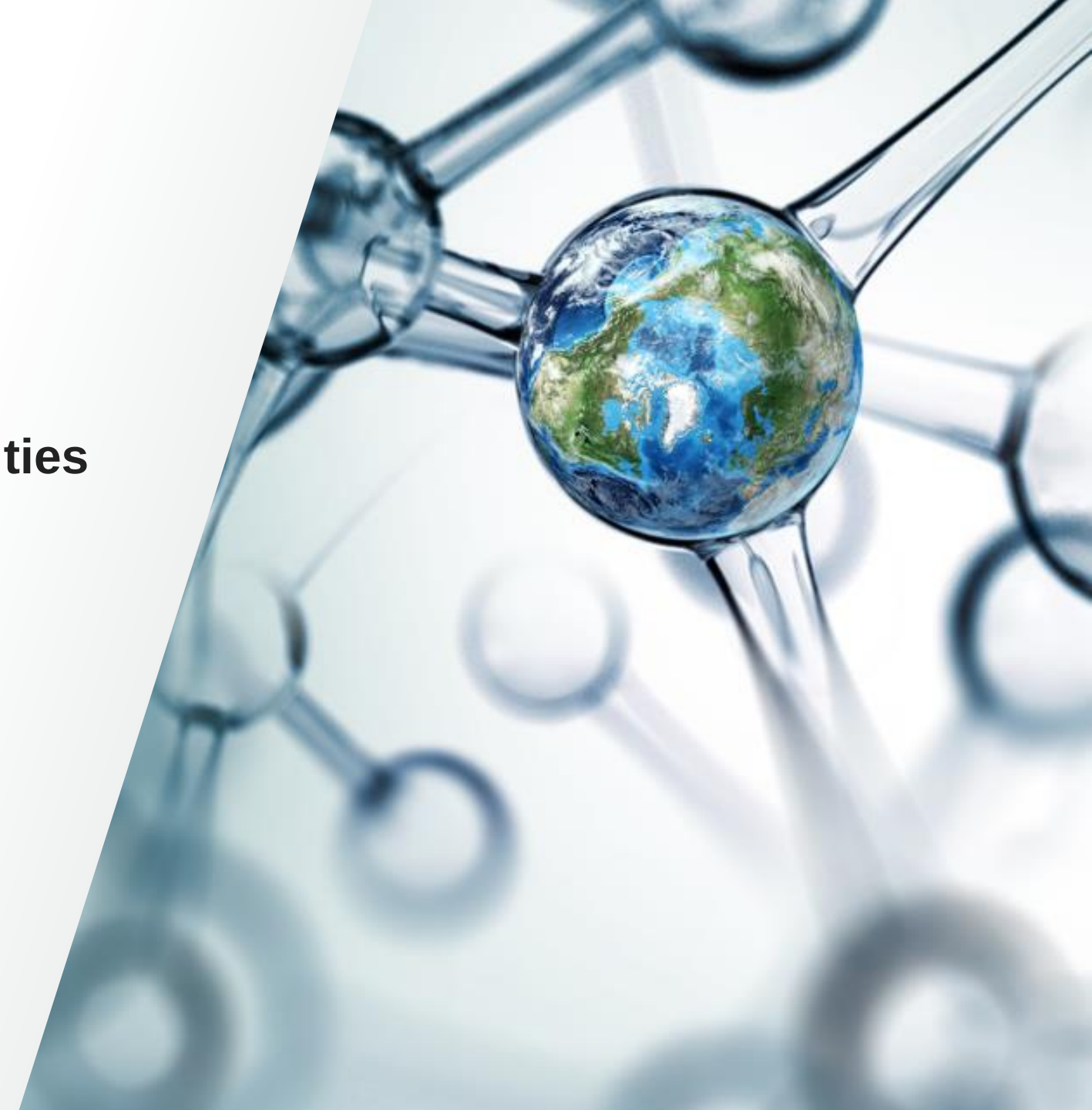
Streamlining workflow from characterization to monitoring of therapeutic oligonucleotides impurities using Orbitrap based LC-HRAM-MS platforms

Hao Yang, PhD

April 11, 2023



The world leader in serving science

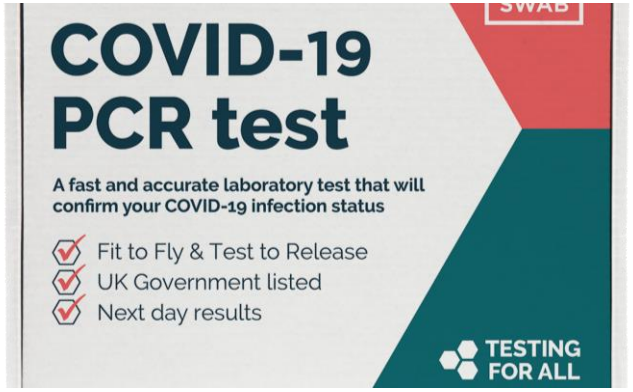
Agenda

- Oligonucleotides – applications and analytical challenges

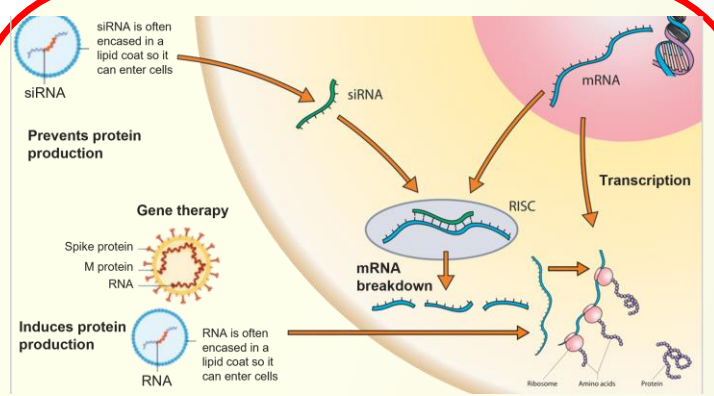
- Application benefits of using
  - Vanquish UHPLC system and consumables
  - Orbitrap Exploris 240 and Orbitrap Exploris MX
  - Biopharma Finder (BPF) 5.0 and Chromeleon (CM) 7.3.1

- Oligonucleotide workflow solution
  - Oligonucleotide characterization using BPF 5.0
  - Impurities monitoring using CM 7.3.1
  - Oligo system performance evaluation test (SET)

Oligonucleotide Applications



- Synthetic DNA primers
- Unmodified, PCR analysis
- Typically, around18 – 30 bases
- High demand



- Therapeutic oligos (e.g., siRNA and ASO)
- Around 20mer in length, heavily modified
- 15 FDA approved drugs*
- miRNAs/sgRNA (up to 120mer)

* https://doi.org/10.2217/frd-2021-0008

- mRNA vaccines
- Up to 5,000 nucleotides
- Sequencing requires digestion
- Associated with LNP and viral vectors for delivery
- mRNA direct sequence mapping application

Therapeutic Oligonucleotides

Target	❑ Single stranded antisense oligos, miRNA/sgRNAs, double stranded siRNAs ❑ Variable length 20 – 120 nucleotides ❑ Heavily modified (e.g., PS, 2'O-Met/F/MOE, LNA, etc.) ❑ Product related impurities (e.g., n-x, n+x, base modifications, etc)
Requirement	❑ Mass confirmation via intact mass deconvolution ❑ Sequence confirmation base-by-base with localization of modification ❑ Relative quantitation of product and its impurities
Challenges	❑ Lack of robust analytical methods to characterize oligonucleotides with increased complexity ❑ Salt adducts can impede accurate mass determination ❑ Lack of software tool for sequence identification, and automated peak annotation with confidence

State-of-the-art Technologies for Oligonucleotide Analysis

Application benefit highlights

Thermo Scientific™ ChromeCare™ Solutions

- Less than 20ppb Na, K, Fe (minimum adduct)
- UHPLC-MS grade

Thermo Scientific™ DNAPac™ RP HPLC Columns

- High resolution separation of short and large oligos
- Wide pH range (1-14) and temperature (up to 100°C)

Thermo Scientific™ Vanquish™ UHPLC System

- Integrated biocompatible system
- Robust and reproducible separation with low baseline noise

Thermo Scientific™ Orbitrap Mass Spectrometer

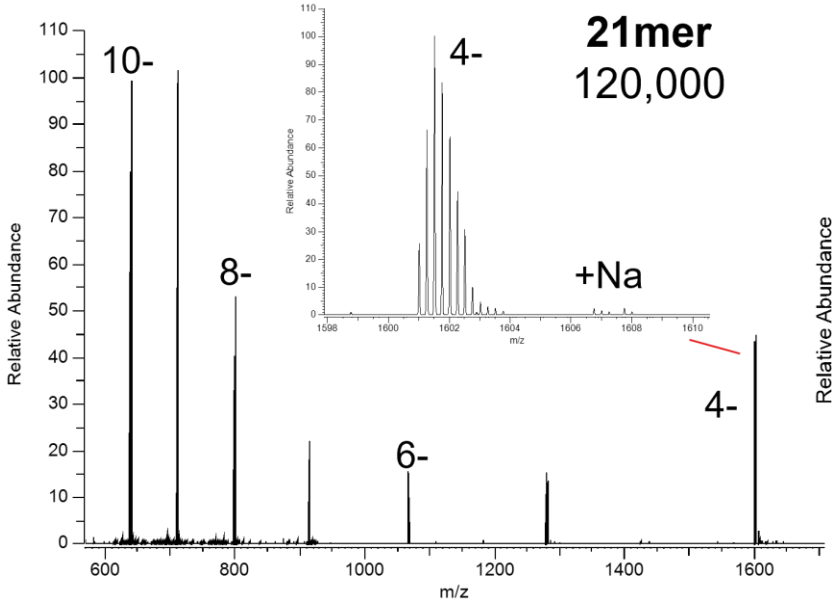
- Operational simplicity with ddMS2 capabilities for Orbitrap Exploris 240, up to 240,000 mass resolution
- Fit-for-purpose with full MS only for Orbitrap Exploris MX

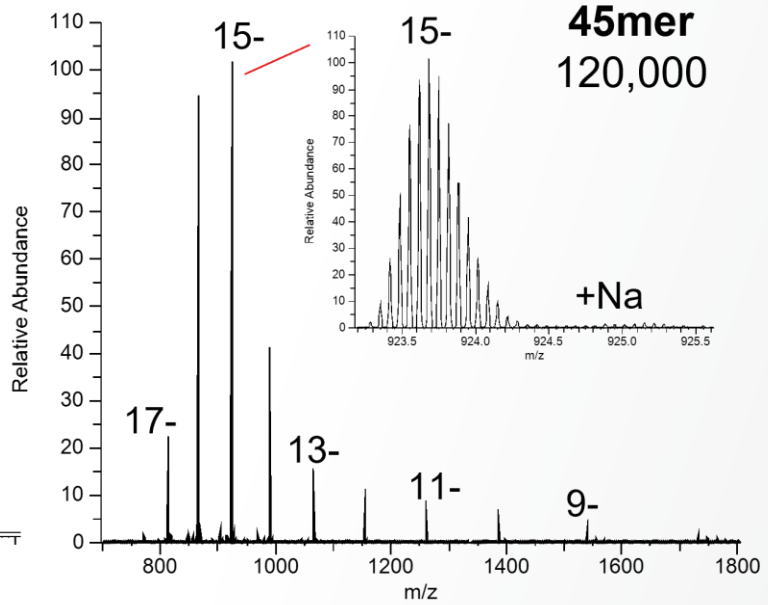
Thermo Scientific™ BioPharma Finder™ 5.0 Thermo Scientific™ Chromeleon™ 7.3.1 Software

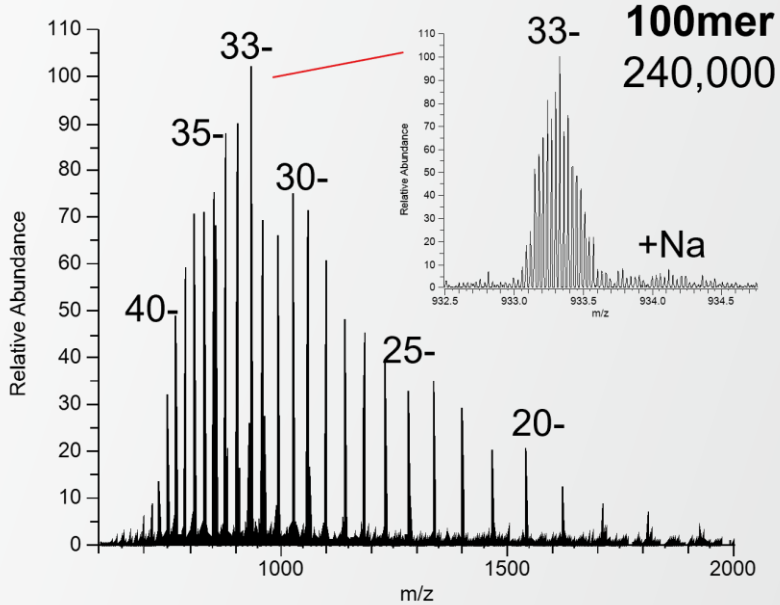
- BPF supports oligo sequencing with customized building blocks
- Automated peak annotation and confirmation
- Compliance ready (CM only)

ChromeCare™ LC-MS Solvents

Importance of clean solvents



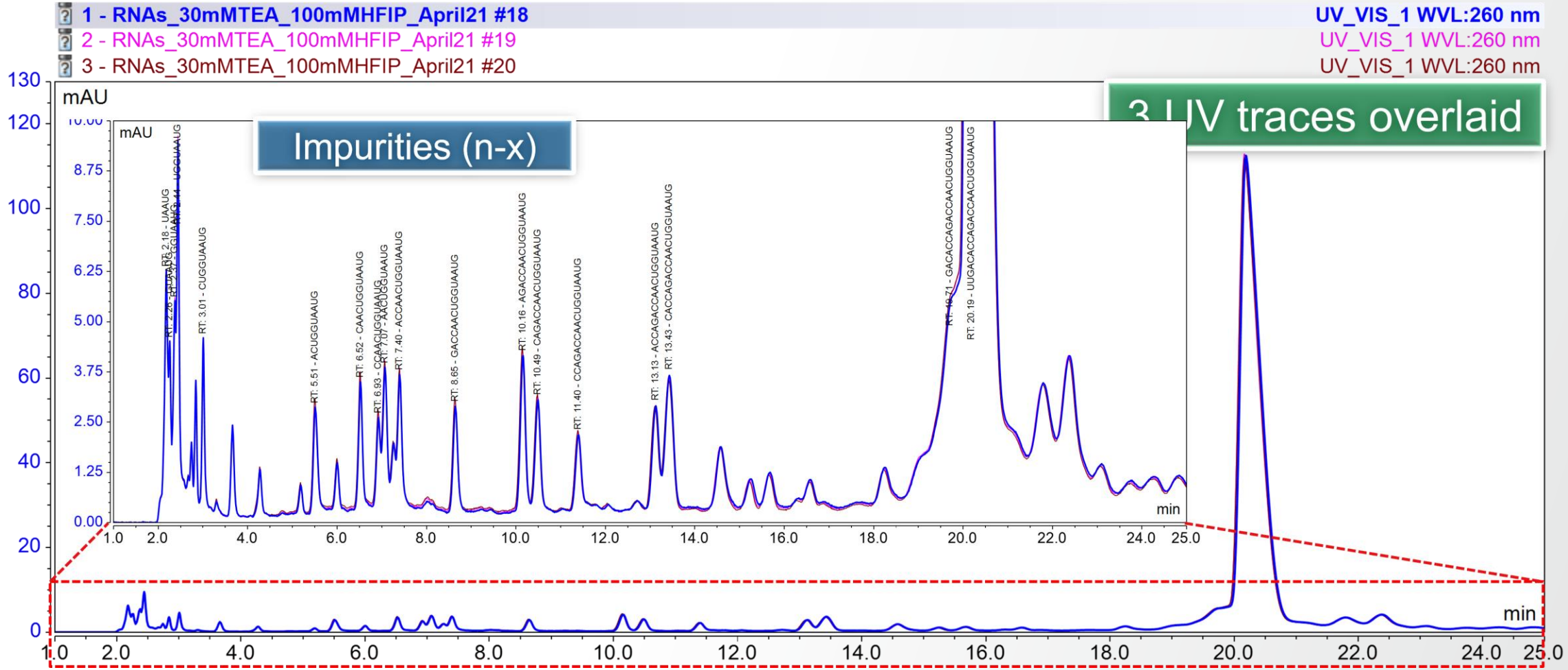




Low metal adduct formation improves quantitation and intact mass deconvolution

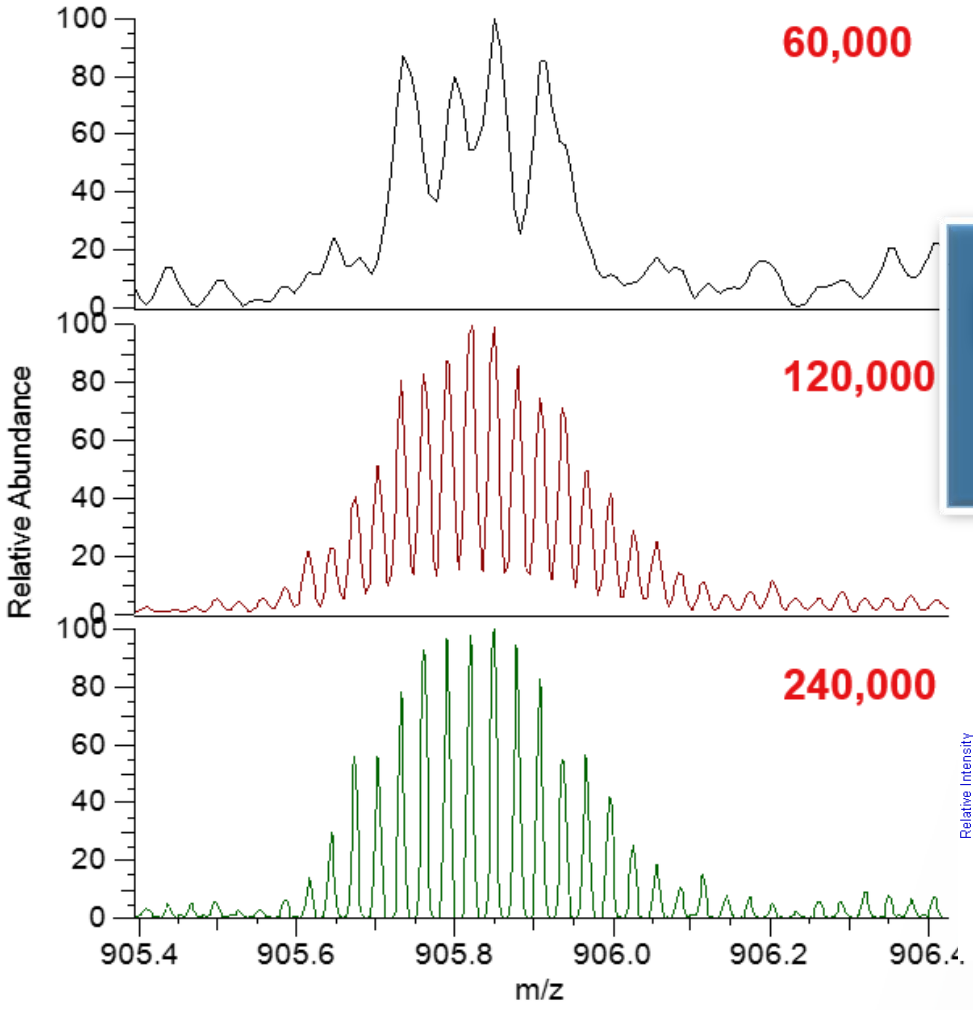
Robust and Reproducible IPRP-LC Separation

Vanquish Horizon UHPLC equipped with DNAPac RP column 2.1 x 250 mm, 4 µm



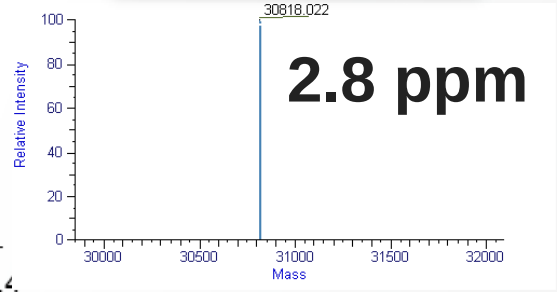
Power of High-Resolution Accurate Mass (HRAM)

34-charge state of 100mer at different resolution settings

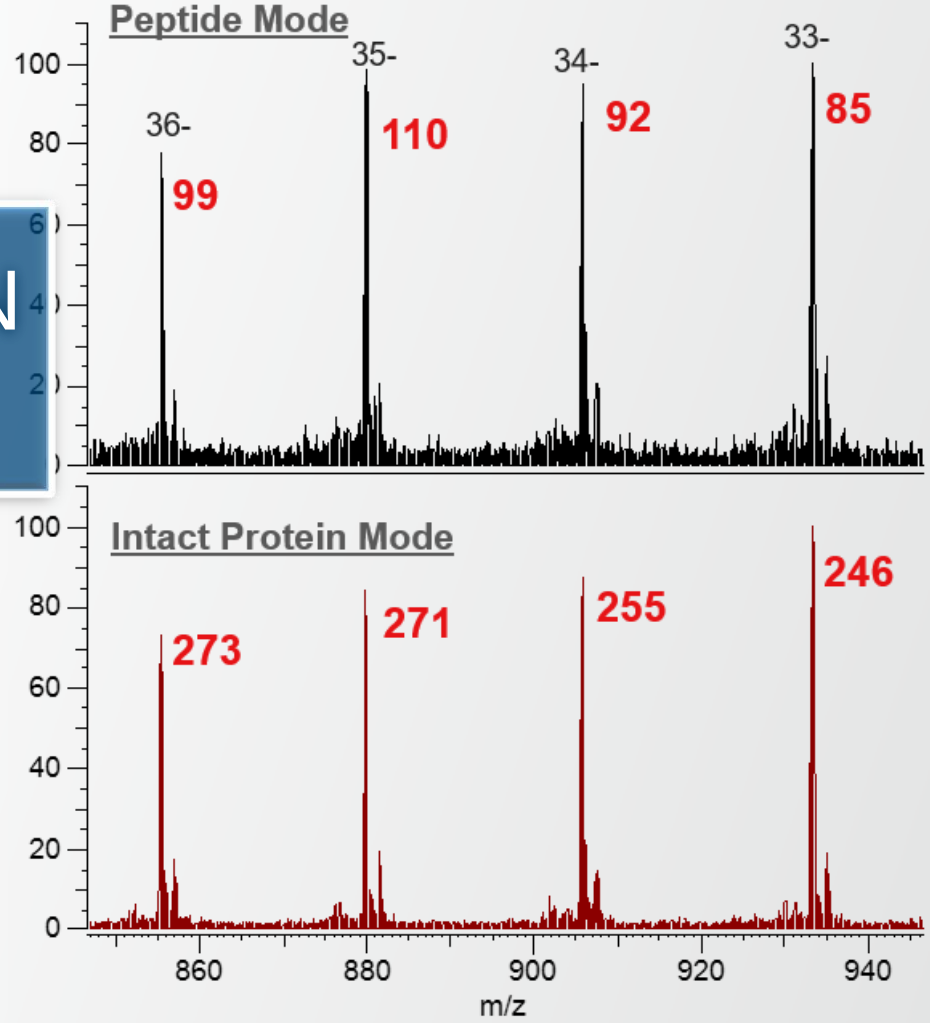


RESOLUTION MATTERS!!!

Deconvolution

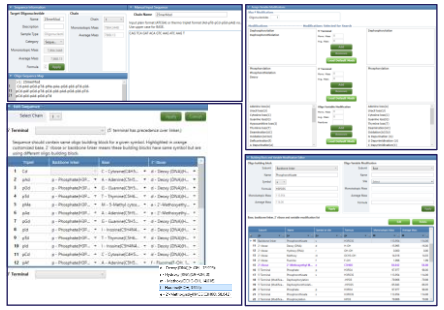


S/N ratios of 100mer in different application modes



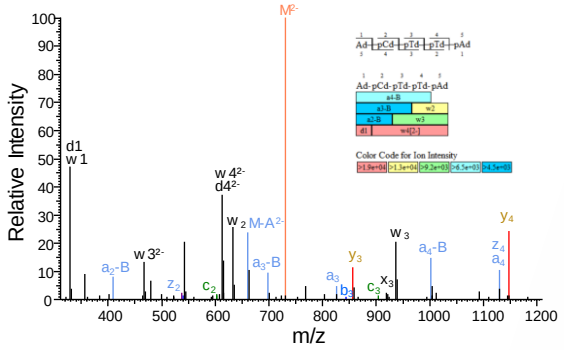
BioPharma Finder Software Offers a Complete Oligonucleotide Analysis

Easy and Flexible Sequence Creation



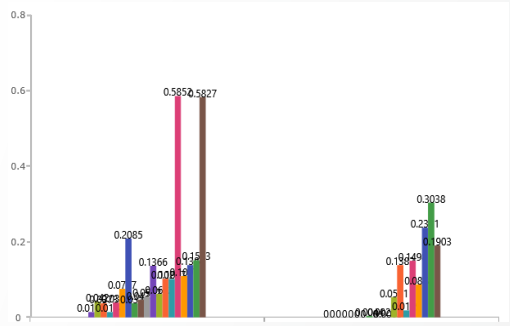
- Support DNA or RNA
- Enter sequence in a plain or triplet format
- Edit sequence to set constant modification
- Assign variable modifications
- Custom building blocks and variable modifications

Automatic MS^2 Annotation



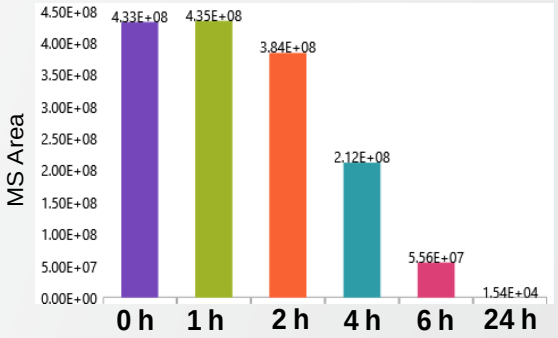
- Fast and confident oligo identification and mapping using ddMS^2 data
- Predicted *vs* annotated experimental MS^2 spectra
- Fragment and sequence coverage maps
- Average structural resolutions (ASR) score

Relative Quantitation of Impurities



- Identification, mapping, and relative quantitation of oligo impurities in one experiment
- Custom list of components for % abundance calculation
- Modification summary table and plot

Comparative Data Analysis



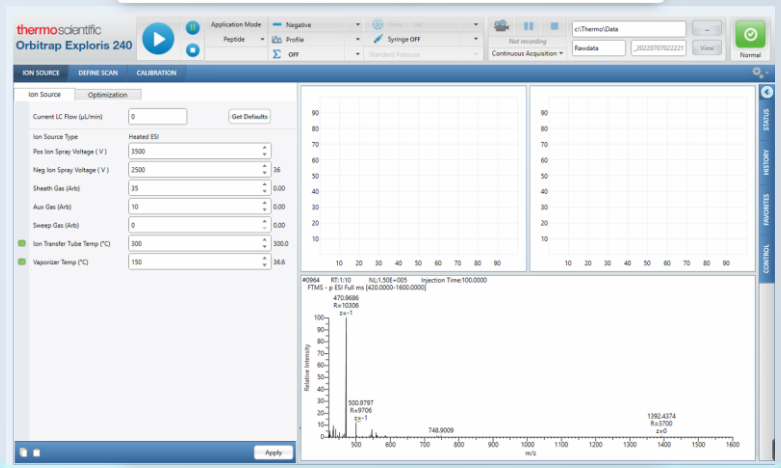
- Comparative analysis of multiple ddMS^2 raw files facilitates method optimization
- An array of features, such as chromatograms, fragment coverage map, MS^2 spectra, MS area, ASR score, can be compared

Chromeleon eCDS

A single software for instrument control, data acquisition, data processing, and report

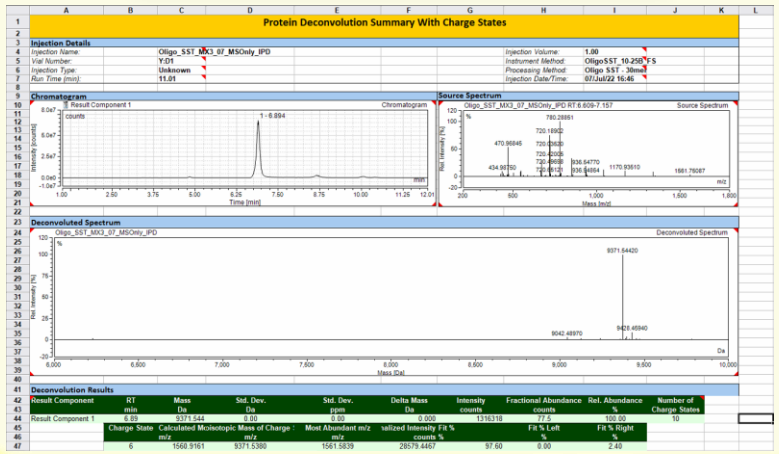
Instrument control

Data acquisition



Compliance ready

Report



Protein Deconvolution Summary With Charge States

Injection Details

Injection Name:	Oligo_SST_MX3_07_MSOnly_IPD	Injection Volume:	1.00
Vial Number:	Y:D1	Instrument Method:	OligoSST_10.250*FS
Injection Type:	Unknown	Processing Method:	Oligo SST - 30mel
Run Time (min):	11.01	Injection Date/Time:	07/Jul/22 16:46

Chromatogram

Result Component 1 — Chromatogram

Source Spectrum

Oligo_SST_MX3_07_MSOnly_IPD RT:6.609-7.157 — Source Spectrum

Deconvoluted Spectrum

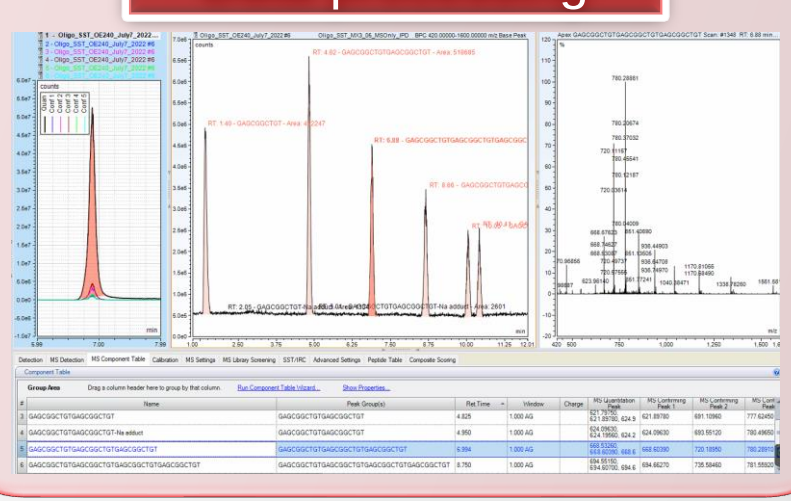
Oligo_SST_MX3_07_MSOnly_IPD — Deconvoluted Spectrum

Deconvolution Results

Result Component	RT min	Mass Da	Std. Dev. Da	Std. Dev. ppm	Delta Mass Da	Intensity counts	Fractional Abundance counts	Rel. Abundance %	Number of Charge States
Result Component 1	6.89	9371.544	1.80	8.62	-0.009	1316318	72.6	100.00	10

Charge State	Calculated Monoisotopic m/z	Mass of Charge : m/z	Most Abundant m/z	Normalized Intensity counts %	Fit %	Fit % Left %	Fit % Right %
6	1560.9161	9371.5380	1561.5839	28579.4467	97.60	0.00	2.40

Data processing



Agenda

- Oligonucleotides – applications and analytical challenges

- Application benefits of using
  - Vanquish UHPLC system and consumables
  - Orbitrap Exploris 240 and MX
  - Biopharma Finder (BPF) 5.0 and Chromeleon (CM) 7.3.1

- Oligonucleotide workflow solution
  - Oligonucleotide characterization using BPF 5.0
  - Impurities monitoring using CM 7.3.1
  - Oligo system performance evaluation test (SET)

Oligonucleotide Workflow Solution

From characterization to monitoring of oligonucleotide impurities using Orbitrap based LC-HRAM-MS platforms

Oligonucleotide characterization

Oligo SET

Thermo Scientific™ Vanquish™ Horizon or Flex UHPLC system

coupled to

Thermo Scientific™ Orbitrap Exploris™ 240 Mass Spectrometer

eWorkflow procedure

- LC & MS acquisition methods
- Injection sequence
- Result view template
- Processing method
- Report template

Oligonucleotide monitoring

Oligo SET

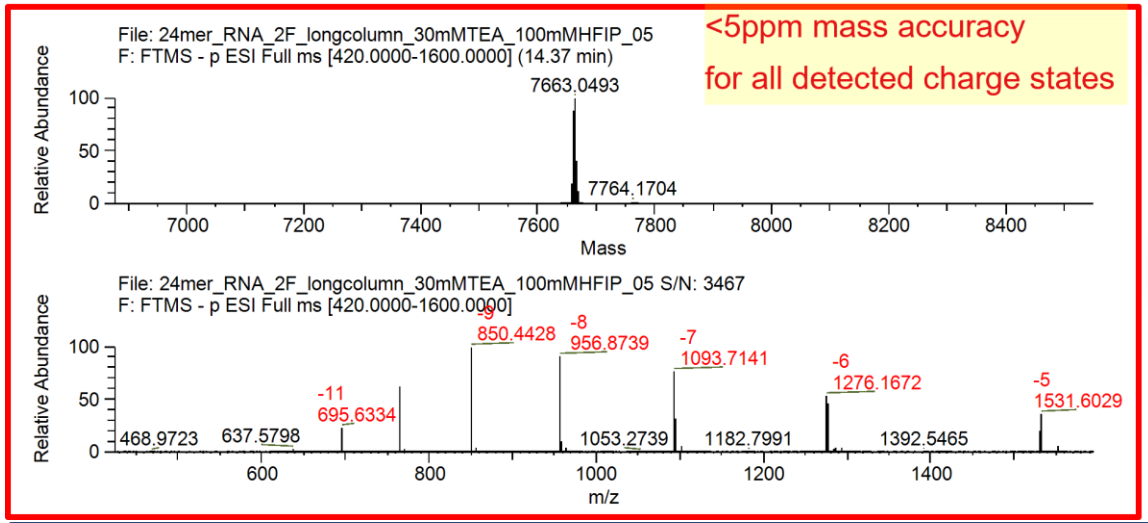
Thermo Scientific™ Vanquish ™ Horizon or Flex UHPLC system

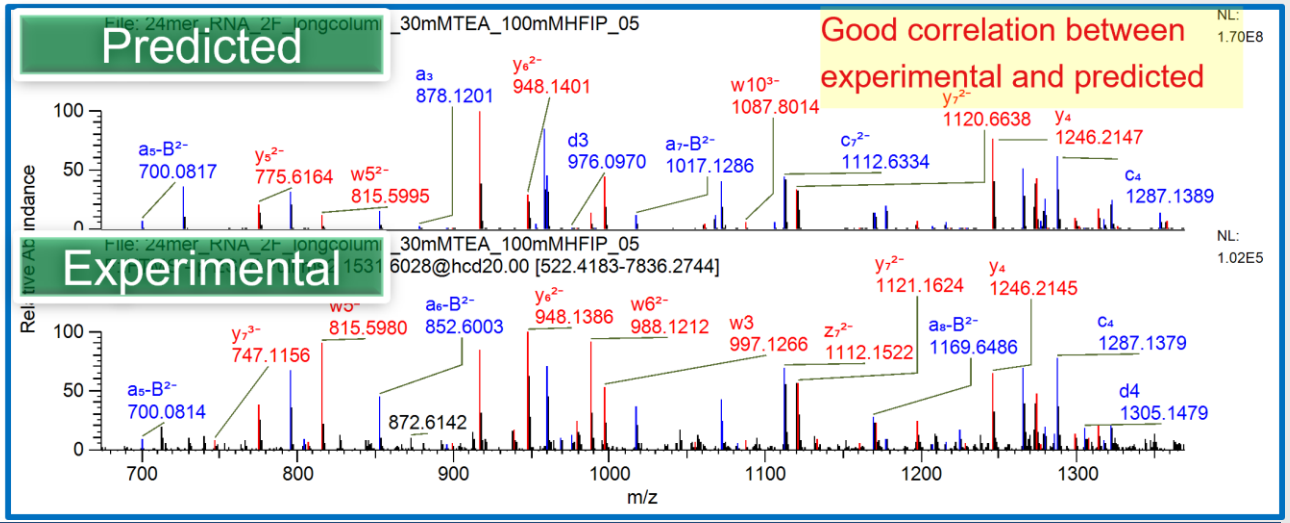
coupled to

Thermo Scientific™ Orbitrap Exploris™ MX Mass Detector

Oligonucleotide Characterization using BPF 5.0

Confident identification based on **Mass Accuracy** and **MS/MS spectra matching to predicted**





Results

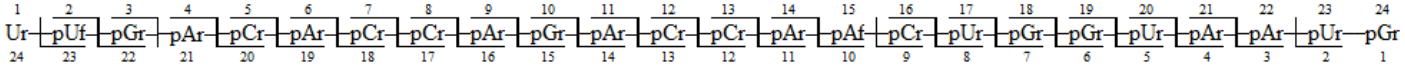
		Level	No.	Identification	Oligo Sequence	Mod	Site	Δ ppm	Conf. Score	Best ASR	ID Type	RT	M/Z	Charge St.	Mono Mass Exp.	Avg Mass Exp.	Theor. Mass
13		C...	14	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-1.97	100.0	1.0	MS2	14.00	918.622	-8	7354.0273	7357.46	7354.0418
14		C...	15	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.37	100.0	1.1	MS2	14.01	1049.996	-7	7354.0244	7357.45	7354.0418
15		C...	16	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.03	100.0	1.1	MS2	14.01	816.440	-9	7354.0269	7357.72	7354.0418
16		C...	26	1:U1-G24 = 7676.0619m(~C13+Oxidation)	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	Oxidation	~C13	-0.70	53.4	1.1	MS2	14.28	852.109	-9	7676.0566	7679.20	7676.0620
17		C...	55	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-3.79	100.0	1.0	MS2	14.36	637.496	-12	7660.0381	7663.72	7660.0671
18		C...	58	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-3.22	100.0	1.0	MS2	14.36	695.633	-11	7660.0425	7663.39	7660.0671
19		C...	60	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.77	100.0	1.0	MS2	14.36	765.298	-10	7660.0459	7663.40	7660.0671
20		C...	68	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.96	100.0	1.0	MS2	14.37	1531.603	-5	7660.0444	7663.41	7660.0671
21		C...	69	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.96	100.0	1.0	MS2	14.37	1276.167	-6	7660.0444	7663.39	7660.0671
22		C...	74	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.26	100.0	1.0	MS2	14.38	850.443	-9	7660.0498	7663.38	7660.0671

Oligonucleotide Characterization using BPF 5.0

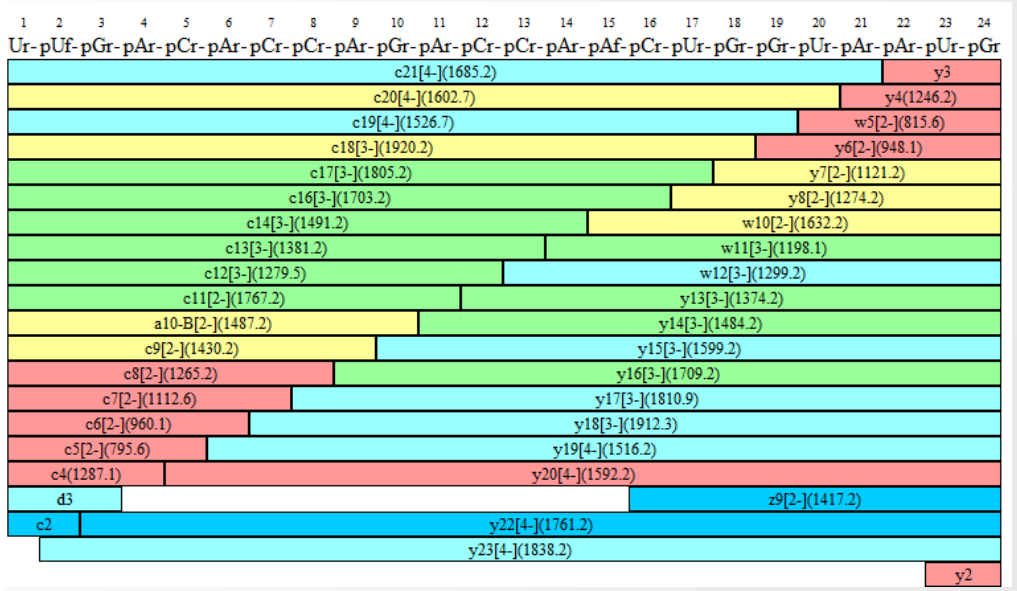
Fragment Coverage Map

Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr (-5)

Average Structural Resolution = 1.0 residues



Average structural resolution (ASR) value of 1.0 means every single nucleotide residue bond has been broken and resulting fragment ions matched the predicted MS/MS spectra



Color Code for Ion Intensity

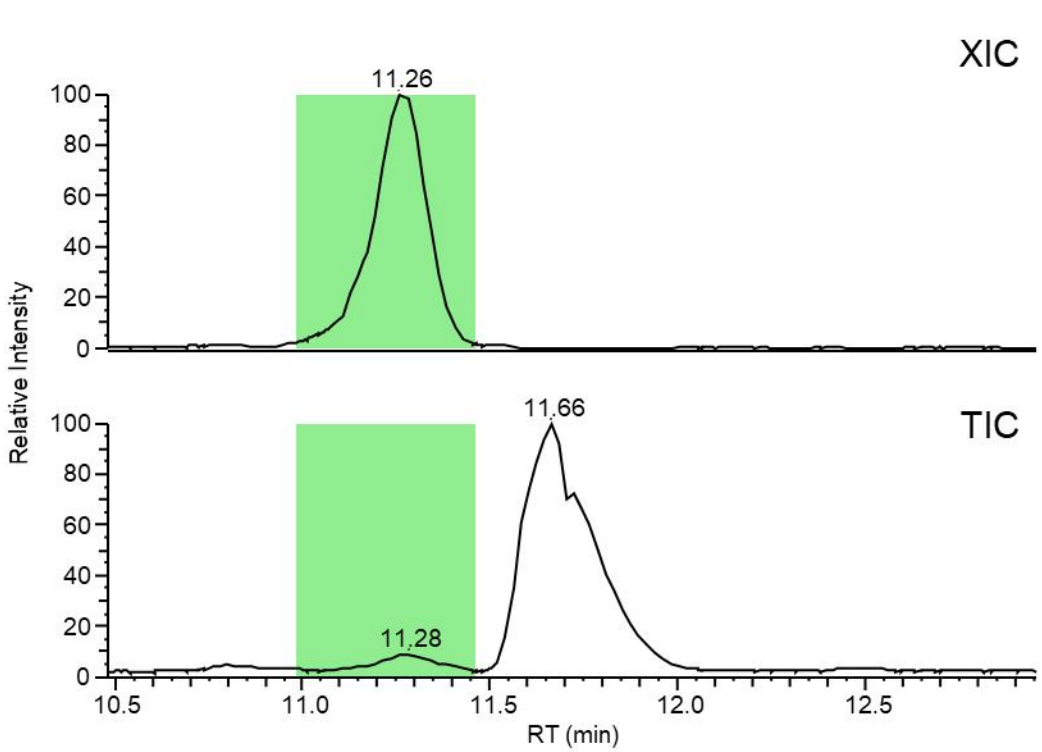
>6.0e+04	>3.5e+04	>2.1e+04	>1.2e+04	>7.2e+03

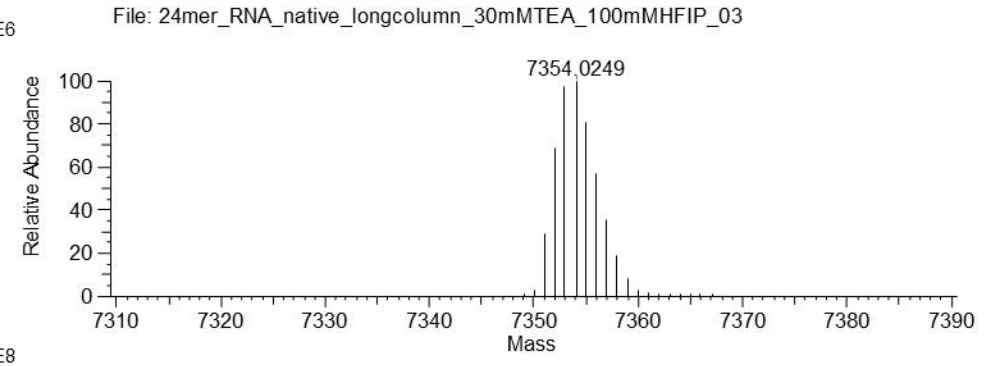
Results

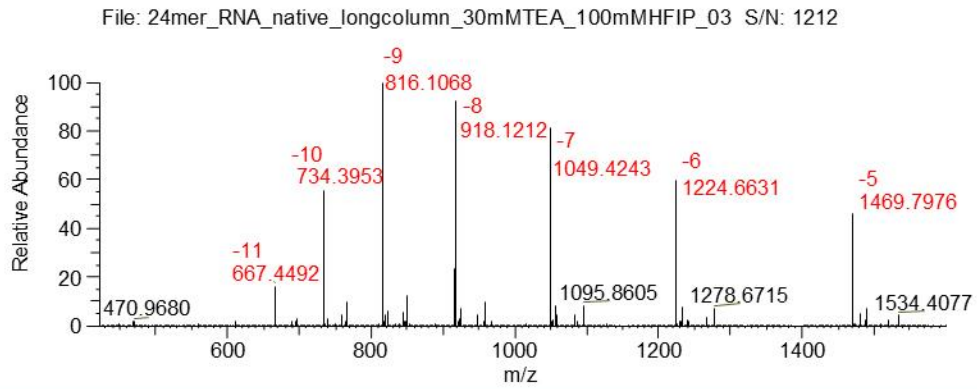
		Level	No.	Identification	Oligo Sequence	Mod	Site	Δ ppm	Conf. Score	Best ASR	ID Type	RT	M/Z	Charge St.	Mono Mass Exp.	Avg Mass Exp.	Theor. Mass
13		C...	14	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-1.97	100.0	1.0	MS2	14.00	918.622	-8	7354.0273	7357.46	7354.0418
14		C...	15	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.37	100.0	1.1	MS2	14.01	1049.996	-7	7354.0244	7357.45	7354.0418
15		C...	16	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.03	100.0	1.1	MS2	14.01	816.440	-9	7354.0269	7357.72	7354.0418
16		C...	26	1:U1-G24 = 7676.0619m(~C13+Oxidation)	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	Oxidation	~C13	-0.70	53.4	1.1	MS2	14.28	852.109	-9	7676.0566	7679.20	7676.0620
17		C...	55	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-3.79	100.0	1.0	MS2	14.36	637.496	-12	7660.0381	7663.72	7660.0671
18		C...	58	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-3.22	100.0	1.0	MS2	14.36	695.633	-11	7660.0425	7663.39	7660.0671
19		C...	60	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.77	100.0	1.0	MS2	14.36	765.298	-10	7660.0459	7663.40	7660.0671
20		C...	68	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.96	100.0	1.0	MS2	14.37	1531.603	-5	7660.0444	7663.41	7660.0671
21		C...	69	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.96	100.0	1.0	MS2	14.37	1276.167	-6	7660.0444	7663.39	7660.0671
22		C...	74	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.26	100.0	1.0	MS2	14.38	850.443	-9	7660.0498	7663.38	7660.0671

High confidence score with low Δppm and low ASR value provides accurate and confident sequence identification and confirmation

Oligonucleotide Characterization using BPF 5.0





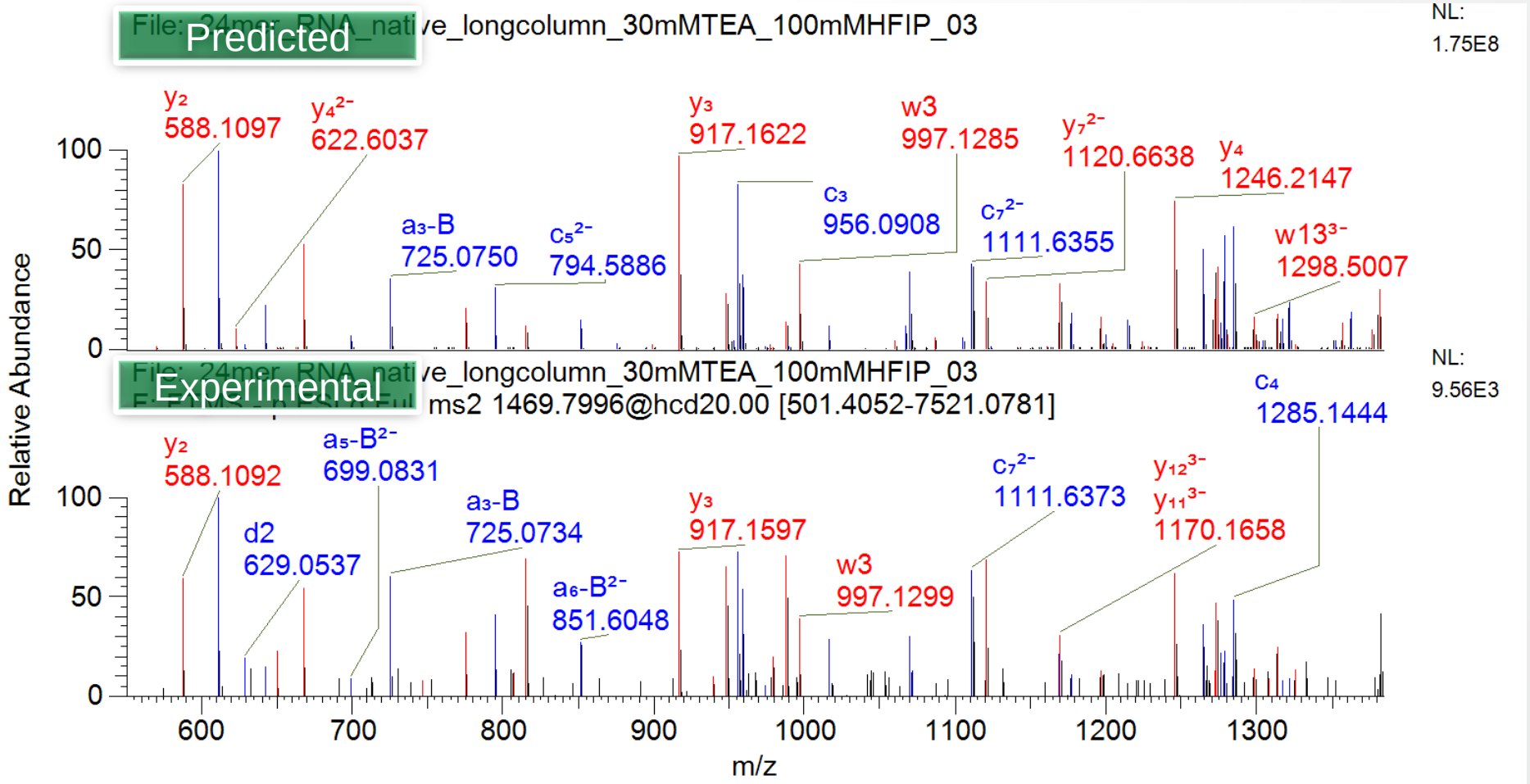


Results

	Identification	Mod	Site	M/Z	Charge St.	Mono Mass Exp.	Avg Mass Exp.	Theor. Mass	Δ ppm	Conf. Score	Best ASR	RT	MS Area
51	1:U1-G24 = 7351.0347m(~C13+RNA_Cytosine triple loss)	RNA_Cytosine triple loss	~C13	1469.797	-5	7351.0225	7354.13	7351.0345	-1.64	99.9	1.0	11.27	414,045.41
52	1:U1-G24 = 7351.0347m(~C13+RNA_Cytosine triple loss)	RNA_Cytosine triple loss	~C13	1049.424	-7	0.0000	7354.12	7351.0347	0.00	99.9	1.0	11.27	753,516.56
53	1:U1-G24 = 7351.0347m(~C13+RNA_Cytosine triple loss)	RNA_Cytosine triple loss	~C13	1224.663	-6	7351.0215	7354.13	7351.0345	-1.77	99.9	1.0	11.27	553,913.81
54	1:U1-G24 = 7351.0347m(~C13+RNA_Cytosine triple loss)	RNA_Cytosine triple loss	~C13	918.121	-8	7351.0283	7354.17	7351.0345	-0.84	99.9	1.0	11.27	815,708.62
55	1:U1-G24 = 7351.0347m(~C13+RNA_Cytosine triple loss)	RNA_Cytosine triple loss	~C13	734.395	-10	7351.0225	7354.17	7351.0345	-1.64	99.9	1.0	11.27	457,254.94
56	1:U1-G24 = 7351.0347m(~C13+RNA_Cytosine triple loss)	RNA_Cytosine triple loss	~C13	816.107	-9	7351.0273	7354.12	7351.0345	-0.97	99.9	1.0	11.27	868,029.06

Provide base-by-base sequence information and localization of modifications

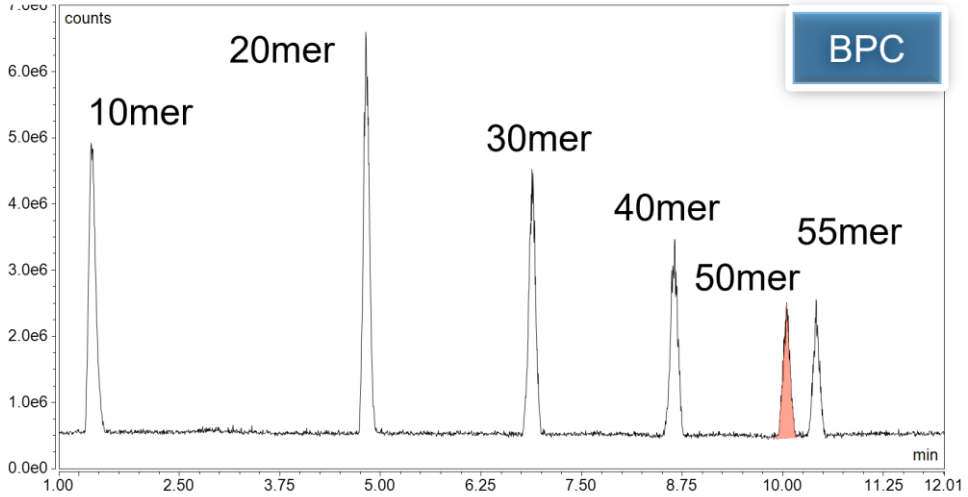
Oligonucleotide Characterization using BPF 5.0

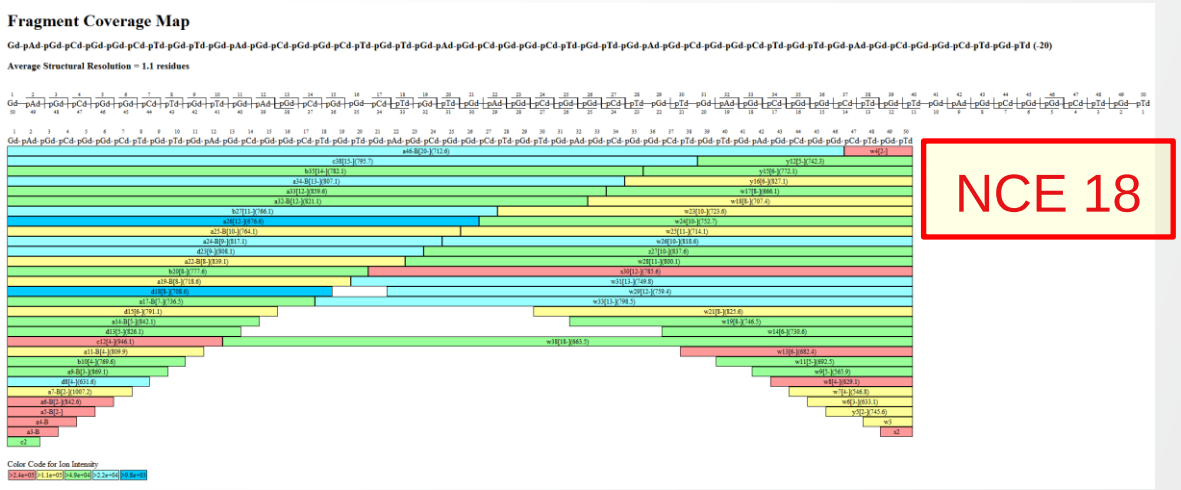


Provide base-by-base sequence information and localization of modifications

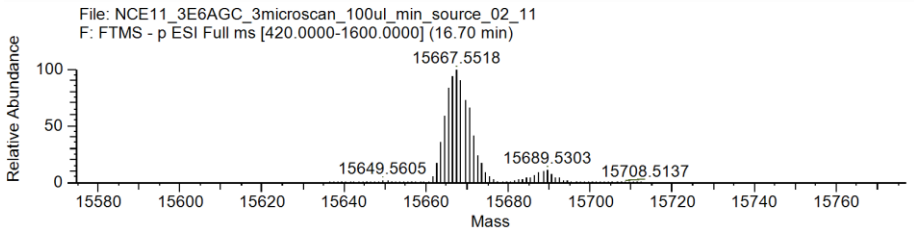
NCE Energy Optimization

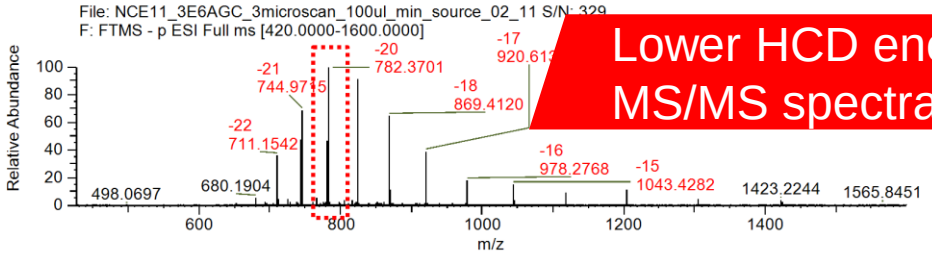
Higher the NCE energy, more internal fragmentation, not ideal for long oligos





NCE 18

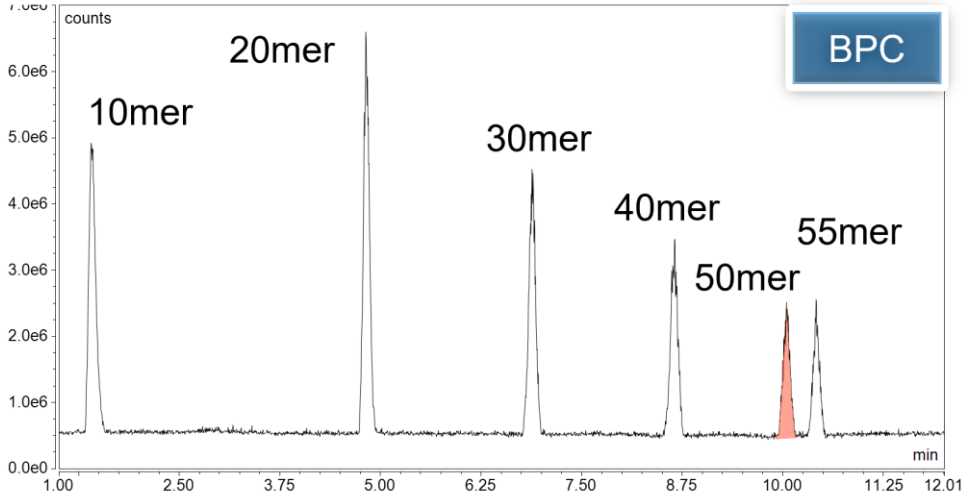


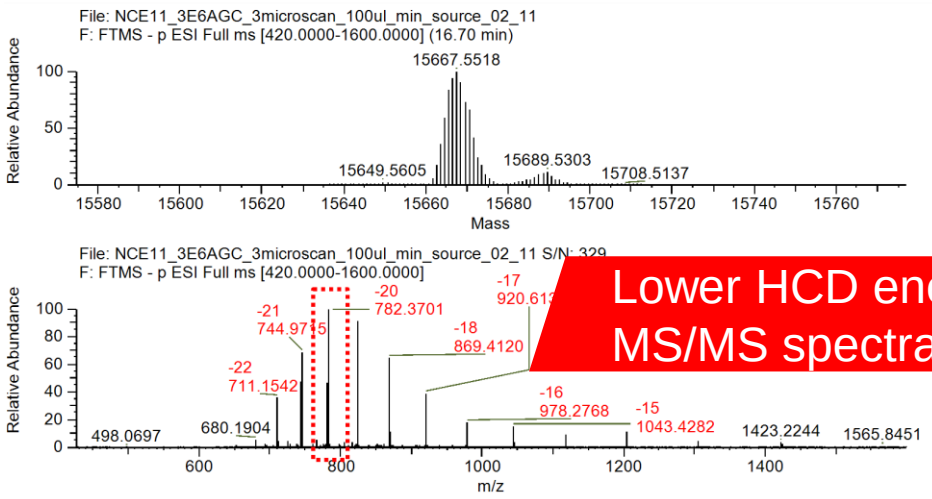


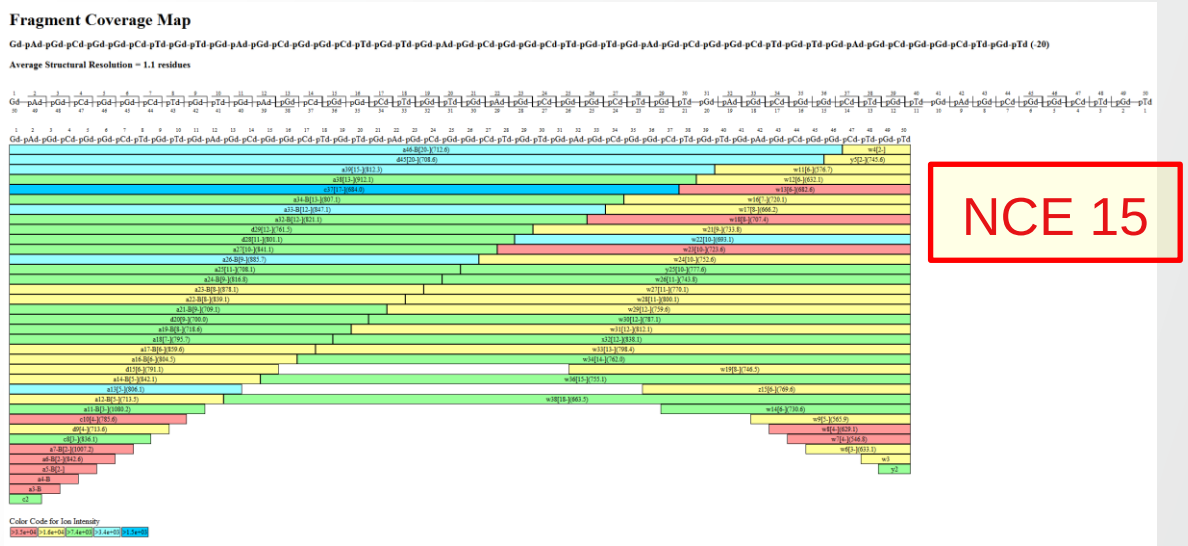
Lower HCD energy gives rich MS/MS spectra for long oligos

NCE Energy Optimization

Higher the NCE energy, more internal fragmentation, not ideal for long oligos





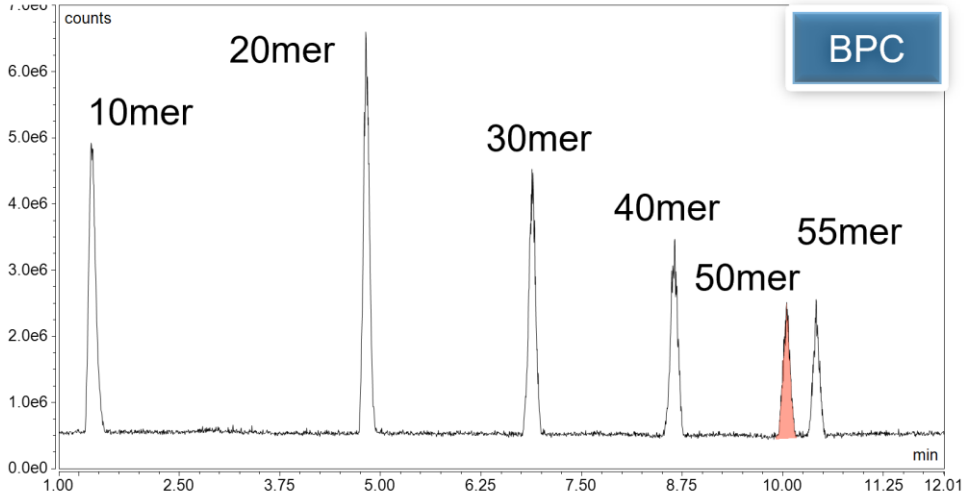


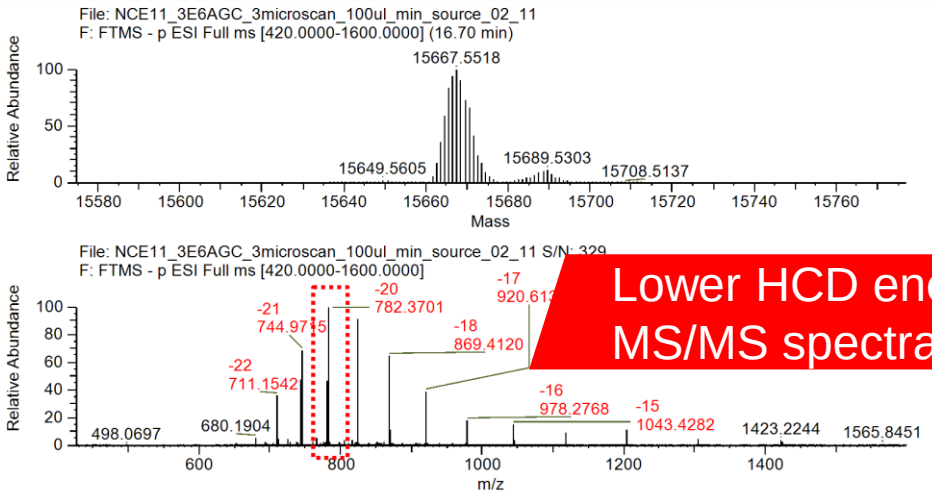
NCE 15

Lower HCD energy gives rich MS/MS spectra for long oligos

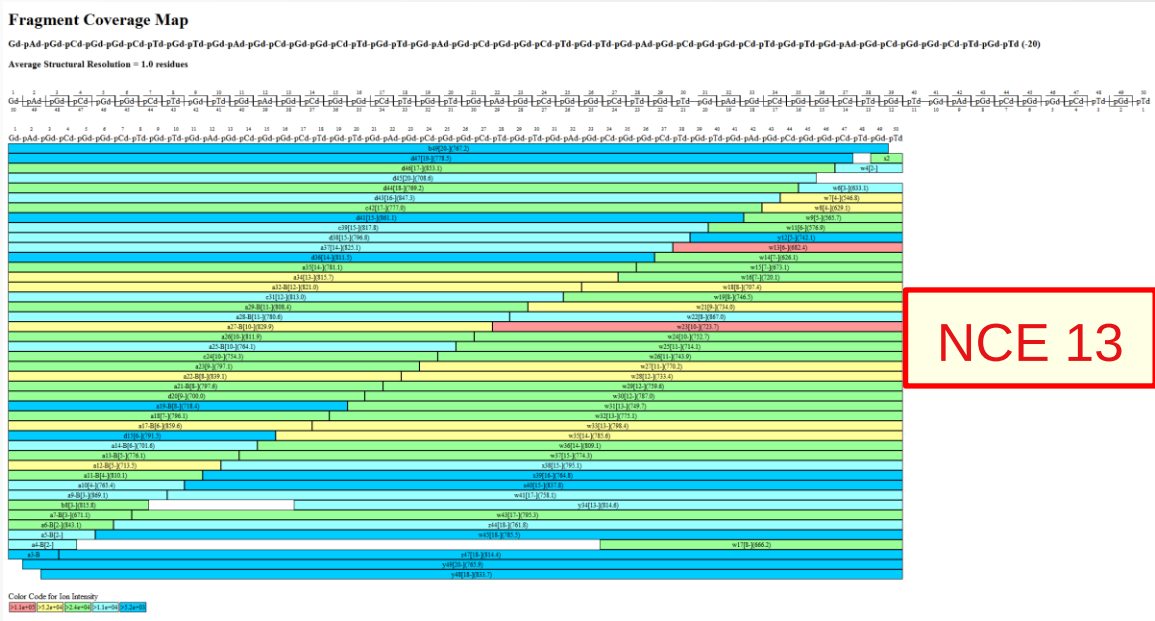
NCE Energy Optimization

Higher the NCE energy, more internal fragmentation, not ideal for long oligos





Lower HCD energy gives rich MS/MS spectra for long oligos



NCE 13

Create Oligonucleotide Component List for Monitoring

Use **filters** to create an oligonucleotide component list in BPF 5.0

Results

		Level	No.	Identification	Oligo Sequence	Mod	Site	Δ ppm	Conf. Score	Best ASR	RT	M/Z	Charge St.	Mono Mass Exp.
1	☑	Comp	2	1:A15-G24 = 3185.4570m[nonspecific]	pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-1.89	100.0	1.1	4.12	795.606	-4	3185.4509
2	☑	Comp	3	1:A15-G24 = 3185.4570m[nonspecific]	pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.43	100.0	1.0	4.12	636.283	-5	3185.4492
3	☑	Comp	4	1:A15-G24 = 3185.4570m[nonspecific]	pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-1.81	100.0	1.1	4.12	1061.143	-3	3185.4512
4	☑	Comp	5	1:A15-G24 = 3185.4570m[nonspecific]	pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.04	100.0	1.1	4.12	1592.219	-2	3185.4504
5	☑	Comp	6	1:A14-G24 = 3514.5095m[nonspecific]	pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-1.93	100.0	1.0	5.16	877.869	-4	3514.5027
6	☑	Comp	7	1:A14-G24 = 3514.5095m[nonspecific]	pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.83	100.0	1.0	5.16	1170.827	-3	3514.4995
7	☑	Comp	8	1:A14-G24 = 3514.5095m[nonspecific]	pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.69	100.0	1.0	5.16	584.910	-6	3514.5000
8	☑	Comp	10	1:A14-G24 = 3514.5095m[nonspecific]	pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.28	100.0	1.0	5.16	702.094	-5	3514.5015
9	☑	Comp	12	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.50	100.0	1.1	13.99	1225.164	-6	7354.0234
10	☑	Comp	13	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-1.97	100.0	1.3	13.99	1470.398	-5	7354.0273
11	☑	Comp	14	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-1.97	100.0	1.0	14.00	918.622	-8	7354.0273
12	☑	Comp	15	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.37	100.0	1.1	14.01	1049.996	-7	7354.0244
13	☑	Comp	16	1:U2-G24 = 7354.042m[nonspecific]	pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	nonspecific		-2.03	100.0	1.1	14.01	816.440	-9	7354.0269
14	☑	Comp	11	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.01	88.4	1.4	13.98	956.875	-8	7660.0518
15	☑	Comp	55	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-3.79	100.0	1.0	14.36	637.496	-12	7660.0381
16	☑	Comp	58	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-3.22	100.0	1.0	14.36	695.633	-11	7660.0425
17	☑	Comp	60	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.77	100.0	1.0	14.36	765.298	-10	7660.0459
18	☑	Comp	68	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.96	100.0	1.0	14.37	1531.603	-5	7660.0444
19	☑	Comp	69	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.96	100.0	1.0	14.37	1276.167	-6	7660.0444
20	☑	Comp	74	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.26	100.0	1.0	14.38	850.443	-9	7660.0498
21	☑	Comp	75	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.39	100.0	1.0	14.38	1093.714	-7	7660.0488
22	☑	Comp	77	1:U1-G24 = 7660.067m	Ur-pUf-pGr-pAr-pCr-pAr-pCr-pCr-pAr-pGr-pAr-pCr-pCr-pAr-pAf-pCr-pUr-pGr-pGr-pUr-pAr-pAr-pUr-pGr	None		-2.39	100.0	1.0	14.38	956.874	-8	7660.0488

N - 14

N - 13

N - 1

FLP

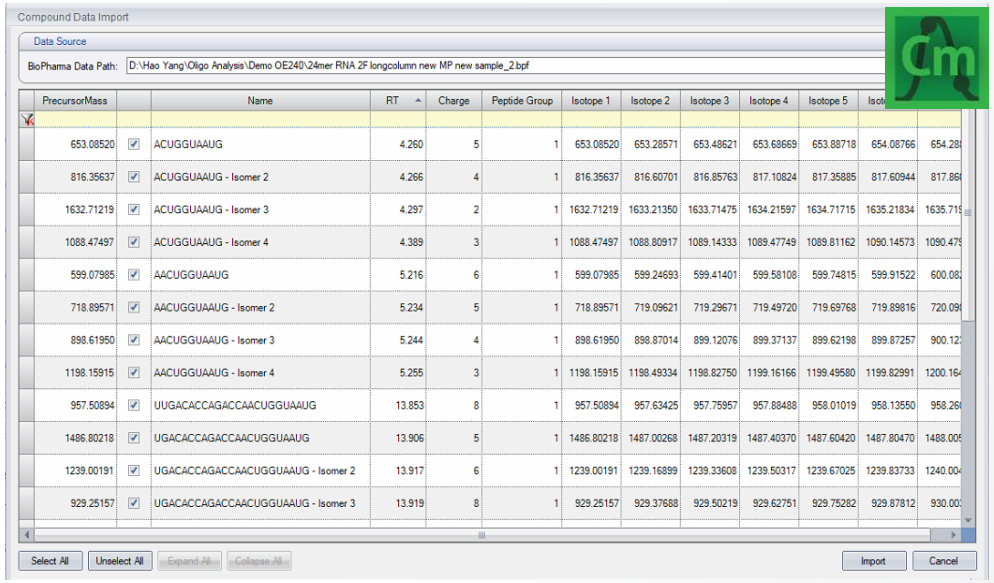
Conf. Score ≥ 80

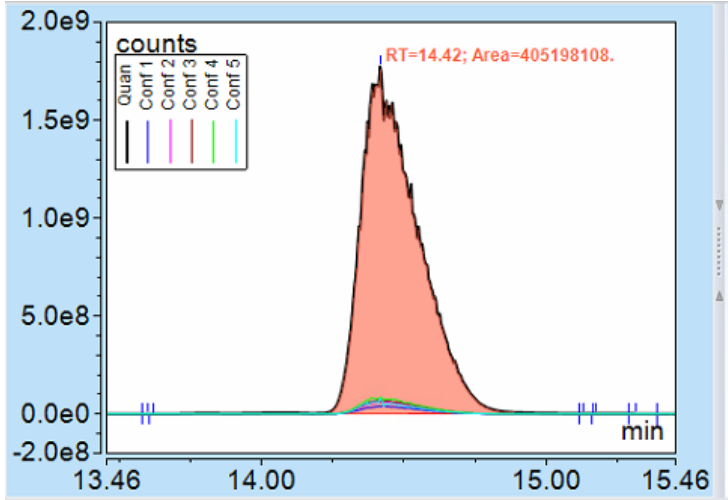
ASR ≤ 1.5

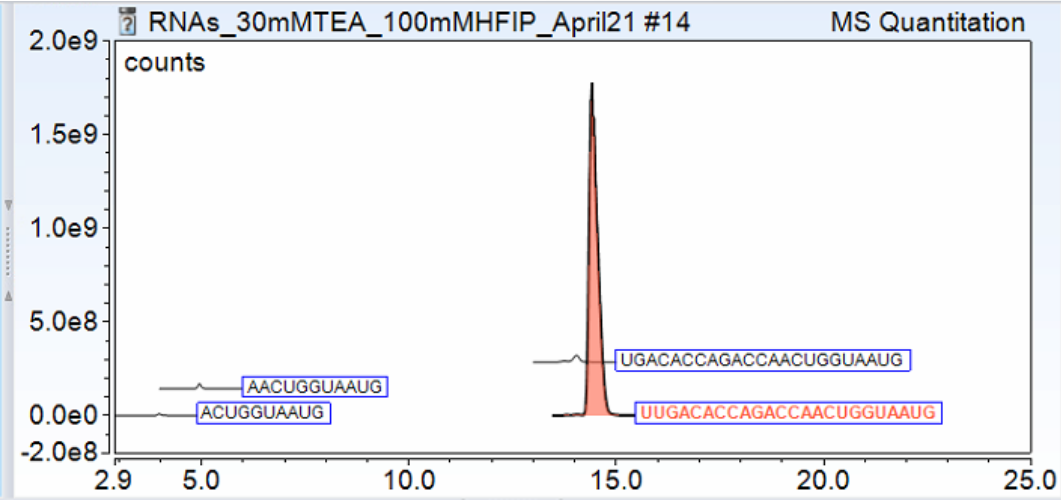
Δppm ≤ 10

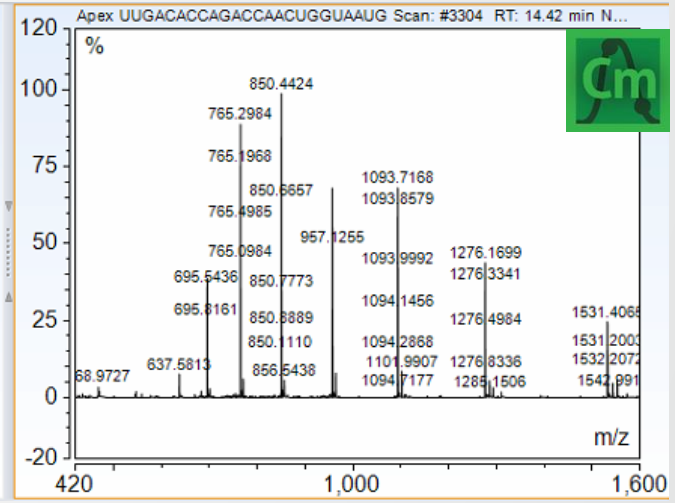
Chromeleon CDS for Oligonucleotide Monitoring

Relative quantitation of oligonucleotide impurities using Chromeleon 7.3.1









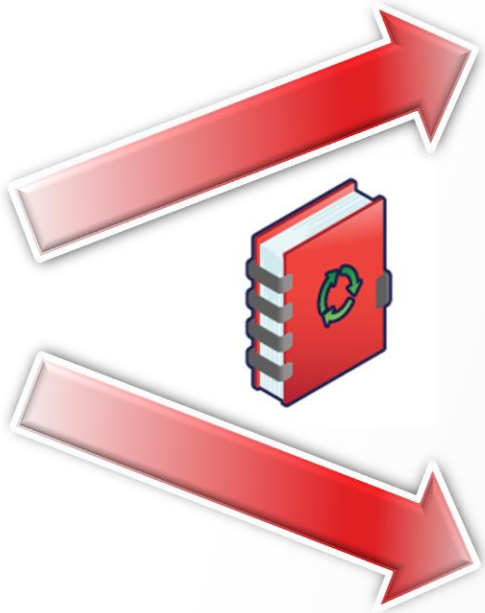
Chromeleon eWorkflow Procedure

Harmonized instrument HW and SW enable seamless method transfer between Orbitrap Exploris platforms

Direct method transfer without physically moving any method files

Characterization setup:

- Vanquish Horizon UHPLC
- Orbitrap Exploris 240 Mass Spectrometer

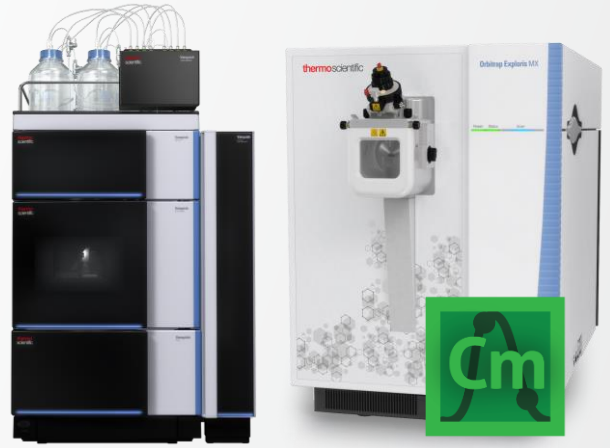


eWorkflow procedure

- LC & MS acquisition methods
- Injection sequence
- Result view template
- Processing method
- Report template

Monitoring setup #1:

- Vanquish Flex UHPLC
- Orbitrap Exploris MX mass detector

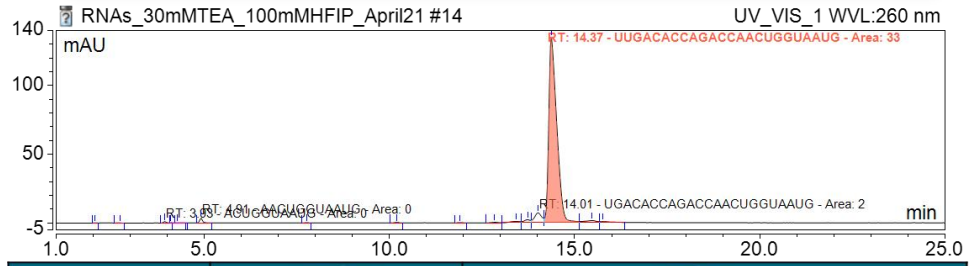


Monitoring setup #2:

- Vanquish Flex UHPLC
- Orbitrap Exploris MX mass detector

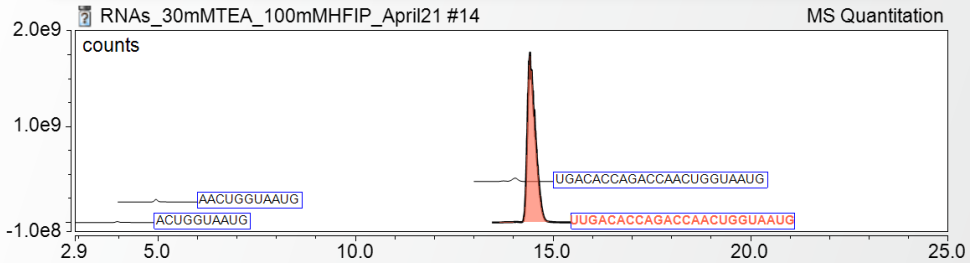
Oligonucleotide Impurities Profiling

LC-UV at 260nm



Components	Characterization setup		Monitoring setup #1		Monitoring setup #2	
	% relative	% RSD	% relative	% RSD	% relative	% RSD
FLP	94.4	0.2	94.8	0.1	94.9	0.1
N-1	4.5	2.0	4.2	1.2	4.2	0.5
N-13	0.83	4.4	0.75	4.4	0.74	3.4
N-14	0.26	26.8	0.20	13.1	0.20	7.0

LC-HRAM-MS



Components	Characterization setup		Monitoring setup #1		Monitoring setup #2	
	% relative	% RSD	% relative	% RSD	% relative	% RSD
FLP	97.4	0.1	97.6	0.1	97.7	0.1
N-1	1.7	2.0	1.64	2.7	1.65	4.8
N-13	0.62	8.1	0.54	4.5	0.48	1.1
N-14	0.25	9.1	0.19	5.4	0.16	2.1

* % relative abundances and %RSDs are calculated based on triplicate injections of 24mer RNA with 2'F modifications

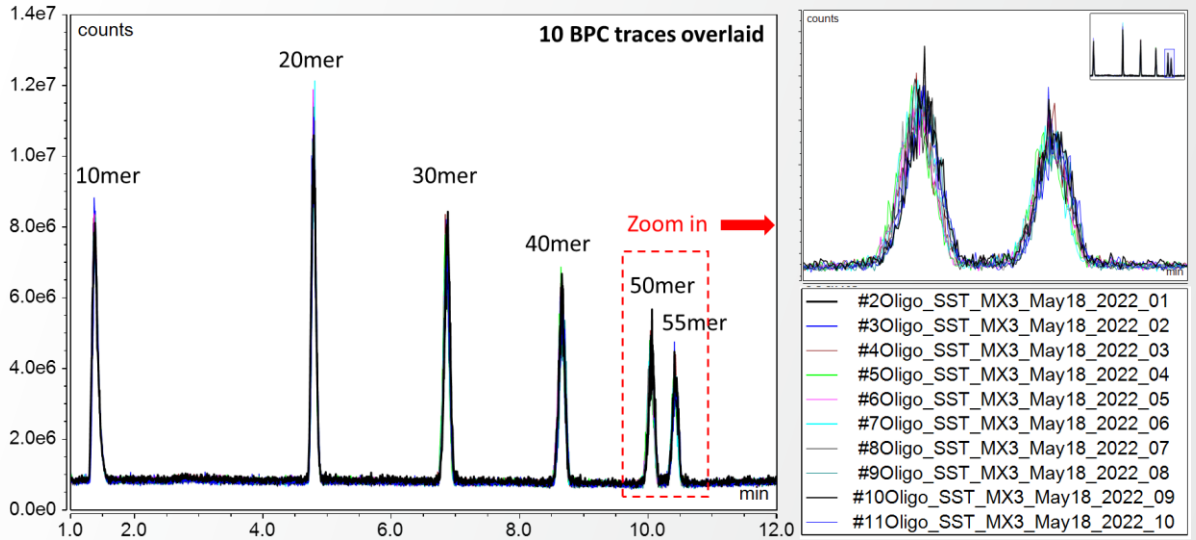
Oligonucleotide SET

System performance tested against pre-defined acceptance criteria

Performance check	Oligonucleotides Sequence	System performance metrics	Acceptance criteria
LC-MS test	1. GAG CGG CTG T **(10mer)** 2. GAG CGG CTG TGA GCG GCT GT **(20mer)** 3. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT **(30mer)** 4. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG T **(40mer)** 5. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GT **(50mer)**	• Retention time reproducibility • Peak area reproducibility • Peak height reproducibility • Peak height range • Peak width at 10% height reproducibility • Peak width at 10% height	• RT %RSD ≤ 2% • Peak area %RSD ≤ 10% • Peak height %RSD ≤ 10% • Peak height range between 2E7 to 2E8 counts • Peak width at 10% height %RSD ≤ 10% • Peak width at 10% height ≤ 0.5 min
Intact mass deconvolution	6. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GTG AGC G **(55mer)**	• Mass accuracy of deconvoluted FLP mass • % Fractional abundance of Na and K adducts	• Mass accuracy of deconvoluted FLP mass ≤ 5 ppm • % Fractional abundance of Na, and K adducts ≤ 10%

Oligonucleotide SET

- Oligonucleotide standards (6 unmodified, ssDNA sequences)
- A sequence containing 10 injections using full MS method only, can be seamlessly deployed using Chromeleon eWorkflow procedure
- Evaluates LC and MS instrument performance related metrics that are important for oligonucleotide applications based on pre-defined acceptance criteria with pass or fail status



LC-MS System Performance Metrics for Oligonucleotide Analysis

Sequence Details					
Name:	Oligo_SST_MX3_May18_2022	RT %CV	< or =	2%	18/May/22 13:52:53
Directory:	Oligos	Peak Area %CV	< or =	10%	Administrator
Data Vault:	ChromeleonLocal	Min Peak Height	> or =	2.00E+07	19/May/22 22:24:47
No. of Injections:	12	Max Peak Height	< or =	2.00E+08	Administrator
		Max. Peak Width 10%	< or =	0.5	
		Peak Width 10% Heigh	< or =	10%	

Peak Summary

Oligonucleotide	Avg. RT (min)	%CV (RT)	%CV (Area)	Min. Peak Height (counts/min)	Max. Peak Height (counts/min)	%CV (Peak Width 10% Height)	Max. Peak Width 10% Height (min)	Pass or Fail
10mer	1.38	0.55%	3.16%	4.23E+07	4.76E+07	1.45%	0.24	Pass
20mer	4.79	0.18%	1.83%	1.10E+08	1.26E+08	2.24%	0.18	Pass
30mer	6.86	0.17%	2.95%	8.18E+07	9.03E+07	1.05%	0.23	Pass
40mer	8.66	0.09%	0.92%	7.68E+07	8.63E+07	1.63%	0.27	Pass
50mer	10.05	0.12%	1.09%	6.69E+07	7.26E+07	1.67%	0.30	Pass
55mer	10.42	0.11%	0.99%	4.36E+07	4.81E+07	2.55%	0.28	Pass

Oligonucleotide SET

System performance tested against pre-defined acceptance criteria

Performance check	Oligonucleotides Sequence	System performance metrics	Acceptance criteria
LC-MS test	1. GAG CGG CTG T **(10mer)** 2. GAG CGG CTG TGA GCG GCT GT **(20mer)** 3. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT **(30mer)** 4. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG T **(40mer)** 5. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GT **(50mer)**	• Retention time reproducibility • Peak area reproducibility • Peak height reproducibility • Peak height range • Peak width at 10% height reproducibility • Peak width at 10% height	• RT %RSD ≤ 2% • Peak area %RSD ≤ 10% • Peak height %RSD ≤ 10% • Peak height range between 2E7 to 2E8 counts • Peak width at 10% height %RSD ≤ 10% • Peak width at 10% height ≤ 0.5 min
Intact mass deconvolution	6. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GTG AGC G **(55mer)**	• Mass accuracy of deconvoluted FLP mass • % Fractional abundance of Na and K adducts	• Mass accuracy of deconvoluted FLP mass ≤ 5 ppm • % Fractional abundance of Na, and K adducts ≤ 10%

Oligonucleotide SET

- Oligonucleotide standards (6 unmodified, ssDNA sequences)
- A sequence containing 10 injections using full MS method only, can be seamlessly deployed using Chromeleon eWorkflow procedure
- Evaluates LC and MS instrument performance related metrics that are important for oligonucleotide applications based on pre-defined acceptance criteria with pass or fail status

Deconvoluted Mass Identification

Sequence Details			
Name:	Oligo_SST_OE240_July7_2022		06/Jul/22 17:38:06
Directory:	Oligos		Administrator
Data Vault:	ChromeleonLocal		19/Jul/22 18:09:39
No. of Injections:	12		Administrator

Deconvoluted mass overview

Component 1

Inj. No.	Oligonucleotide	Position	TargetAccuracy ppm	ExpectedMass Da	Component Identification	Measured Mass Da	Delta Mass ppm	Pass or Fail
6	ST_MX3_05_MSC	Y:D1	10.0	3082.5493	Full length product	3082.5444	1.6	Pass
7	ST_MX3_06_MSC	Y:D1	10.0	6227.0543	Full length product	6227.0451	1.5	Pass
8	ST_MX3_07_MSC	Y:D1	10.0	9371.5593	Full length product	9371.5442	1.6	Pass
9	ST_MX3_08_MSC	Y:D1	10.0	12516.0643	Full length product	12516.0475	1.3	Pass
10	ST_MX3_08_MSC	Y:D1	10.0	15660.5693	Full length product	15660.5436	1.6	Pass
11	ST_MX3_08_MSC	Y:D1	10.0	17249.8309	Full length product	17249.7875	2.5	Pass

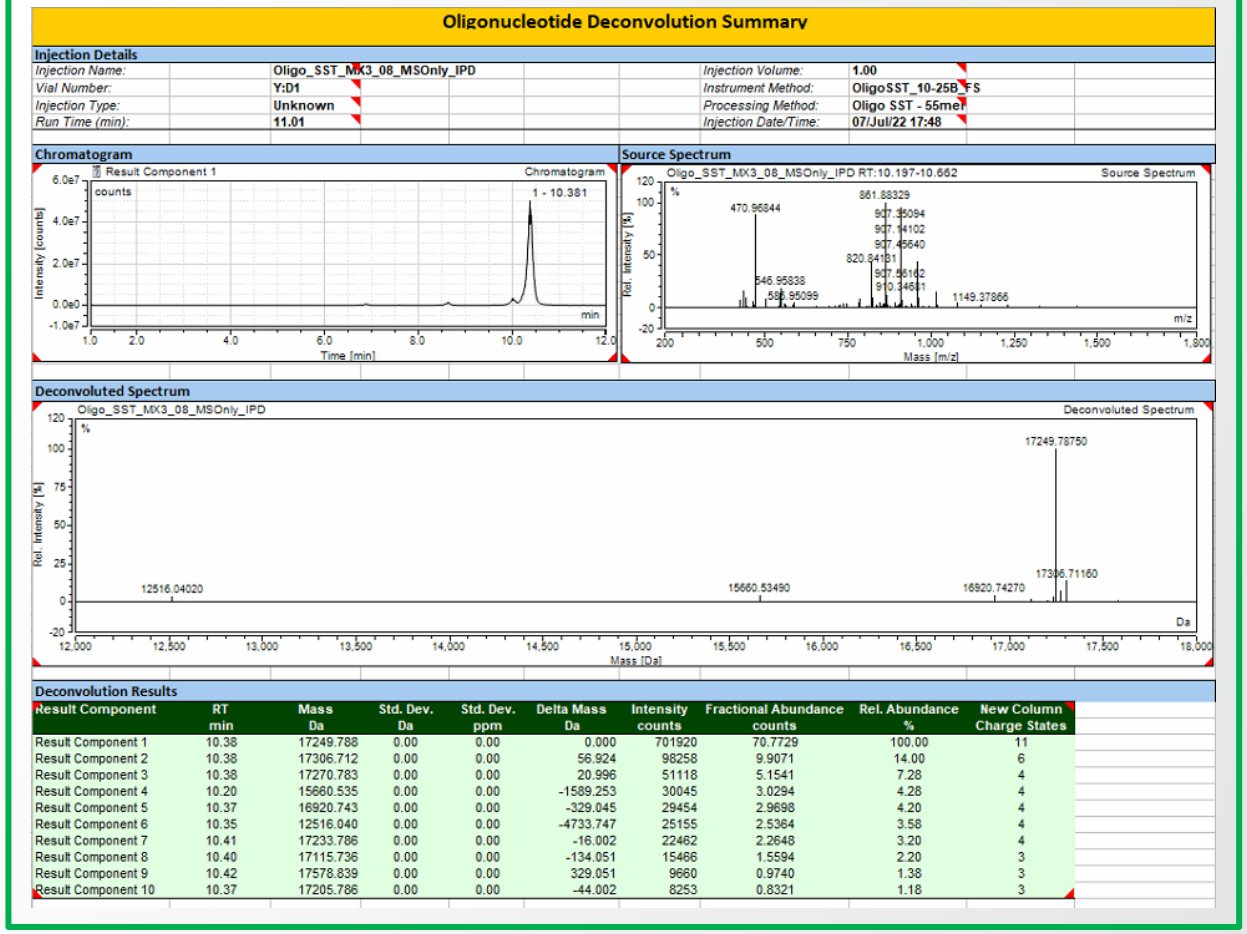
Oligonucleotide SET

System performance tested against pre-defined acceptance criteria

Performance check	Oligonucleotides Sequence	System performance metrics	Acceptance criteria
LC-MS test	1. GAG CGG CTG T **(10mer)** 2. GAG CGG CTG TGA GCG GCT GT **(20mer)** 3. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT **(30mer)** 4. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG T **(40mer)** 5. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GT **(50mer)**	• Retention time reproducibility • Peak area reproducibility • Peak height reproducibility • Peak height range • Peak width at 10% height reproducibility • Peak width at 10% height	• RT %RSD ≤ 2% • Peak area %RSD ≤ 10% • Peak height %RSD ≤ 10% • Peak height range between 2E7 to 2E8 counts • Peak width at 10% height %RSD ≤ 10% • Peak width at 10% height ≤ 0.5 min
Intact mass deconvolution	6. GAG CGG CTG TGA GCG GCT GTG AGC GGC TGT GAG CGG CTG TGA GCG GCT GTG AGC G **(55mer)**	• Mass accuracy of deconvoluted FLP mass • % Fractional abundance of Na and K adducts	• Mass accuracy of deconvoluted FLP mass ≤ 5 ppm • % Fractional abundance of Na, and K adducts ≤ 10%

Oligonucleotide SET

- Oligonucleotide standards (6 unmodified, ssDNA sequences)
- A sequence containing 10 injections using full MS method only, can be seamlessly deployed using Chromeleon eWorkflow procedure
- Evaluates LC and MS instrument performance related metrics that are important for oligonucleotide applications based on pre-defined acceptance criteria with pass or fail status



Result Component	RT min	Mass Da	Std. Dev. Da	Std. Dev. ppm	Delta Mass Da	Intensity counts	Fractional Abundance counts	Rel. Abundance %	New Column Charge States
Result Component 1	10.38	17249.788	0.00	0.00	0.000	701920	70.7729	100.00	11
Result Component 2	10.38	17306.712	0.00	0.00	56.924	98258	9.9071	14.00	6
Result Component 3	10.38	17270.783	0.00	0.00	20.996	51118	5.1541	7.28	4
Result Component 4	10.20	15660.535	0.00	0.00	-1589.253	30045	3.0294	4.28	4
Result Component 5	10.37	16920.743	0.00	0.00	-329.045	29454	2.9698	4.20	4
Result Component 6	10.35	12516.040	0.00	0.00	-4733.747	25155	2.5364	3.58	4
Result Component 7	10.41	17233.786	0.00	0.00	-16.002	22462	2.2648	3.20	4
Result Component 8	10.40	17115.736	0.00	0.00	-134.051	15466	1.5594	2.20	3
Result Component 9	10.42	17578.839	0.00	0.00	329.051	9660	0.9740	1.38	3
Result Component 10	10.37	17205.786	0.00	0.00	-44.002	8253	0.8321	1.18	3

Summary of Benefits

- BioPharma Finder 5.0 software provides confident impurity identification base-by-base with localization of modifications
- Orbitrap Exploris 240 mass spectrometer and Orbitrap Exploris MX detector can achieve comparable quantitative performance for oligo impurities profiling
- Chromeleon eWorkflow procedure facilitates direct method transfer between Orbitrap Exploris 240 mass spectrometer and Orbitrap Exploris MX mass detectors for consistent quantitation of oligonucleotide impurities
- Oligo SET evaluates system performance against a comprehensive set of acceptance criteria that are designed for oligonucleotide applications
- Orbitrap Exploris 240 mass spectrometer provides up to 240,000 mass resolution for isotopic resolution of 100mer
- Vanquish Horizon UHPLC system coupled with a DNAPac RP column provides robust and reproducible separation of oligonucleotides and impurities
- ChromeCare solvents minimize metal adduction formation, and results in accurate mass determination

Thank you