Oligonucleotide Analysis Solutions
From Characterization to QC
Fit-for-purpose LC-MS workflows for oligo analysis:

- **Pages 3-19**: Optimized LC-MS workflows for oligonucleotide analysis from characterization to QC.
  - SEPARATIONS
  - UPLC-MS: QC Analysis with Mass Detection: ACQUITY UPLC H-Class Bio with TUV and ACQUITY QDa Mass Detector
  - UPLC-HRMS: Characterization and High Res Monitoring: ACQUITY UPLC H-Class Bio with TUV and Xevo G2-QS QToF
  - Compliance-Ready Mass Confirmation & Impurity Assessment: BioAccord LC-MS System with TUV
  - UPLC-HDMS: Characterization with Ion Mobility: ACQUITY UPLC H-Class Bio with TUV and SYNAPT G2-Si

- **Pages 20-41**: Additional content on LC-MS workflows.

- **Pages 42-57**: Further detailed information on oligo analysis.

- **Pages 58-63**: Advanced topics and methodologies.

- **Pages 65-76**: Conclusion and future directions.
Tools for Achieving Optimal Oligonucleotide Separations
ACQUITY H-Class Bio UPLC System

- Unrivaled UPLC resolution, sensitivity and throughput for optimum peak clarity and selectivity
- Quaternary Solvent Manager (QSM) with Auto•Blend Plus™ for fully automated multi-solvent blending
- Bio-inert flow path with excellent corrosion resistance - uniquely suited for the high ionic strength aqueous conditions commonly used for biomolecular separations
Oligonucleotide Columns with BEH* Technology

Patented *Bridged Ethyl Hybrid (BEH) Technology delivers N-1 oligo resolution and superior column life at elevated pH values and temperatures

- **ACQUITY UPLC columns** for optimum speed, performance and throughput
  - BEH, C18, 130Å, **1.7µm Particles**:
    - 50 x 2.1 mm, 1.7 µm column
    - 100 x 2.1 mm, 1.7 µm column

- **Xbridge™ HPLC/UHPLC columns** for routine analysis and lab scale purification
  - BEH, C18, 130Å, **2.5µm Particles**:
    - 50 x 2.1 mm, 2.5 µm column
    - 50 x 4.6 mm, 2.5 µm column
    - 50 x 10 mm, 2.5 µm column
Longevity of BEH vs. Silica at High pH & Temperature

Accelerated High pH Stability Test of Competitive Columns

**Analyte: Acenaphthene**

- **BEH Particle Technology**
- **Silica Particles**

Hours in 50 mM TEA, pH 10, 50°C
MassPREP™ Oligonucleotide Standard

Pre-validated oligonucleotide reference material for calibration, troubleshooting and system suitability.

P/N 186004135

Note: Every batch of ACQUITY and XBridge Oligonucleotide column packing material is QC Tested with this standard to ensure performance and consistency.
Ion Pairing Reverse Phase (IP-RP) Chromatography

Reversed-phase interaction **without** Ion-Pairing reagent
- hydrophobicity of the nucleobases

Reversed Phase interaction **with** Ion-Pairing reagent
- Hydrophobicity of the nucleobases + charge-charge interaction (oligo backbone)

TEA$^+$ layer on the column surface

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Ion Pairing Reagents for Oligonucleotide LC-MS Analysis

TEA-HFIP was first ion pairing system for high efficiency LC-MS analysis of oligonucleotides


Waters scientists expanded on these capabilities over the following decade + of research


Range of Ion-Pairing Reagents Commonly Used Today

<table>
<thead>
<tr>
<th>Ion Pairing Agent</th>
<th>Buffering Acid</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triethylammonium</td>
<td>Acetate</td>
<td>TEAA</td>
</tr>
<tr>
<td>Triethylammonium</td>
<td>Bicarbonate</td>
<td>TEAB</td>
</tr>
<tr>
<td>Dimethylbutylammonium</td>
<td>Acetate</td>
<td>DMBAA</td>
</tr>
<tr>
<td>Tributylammonium</td>
<td>Acetate</td>
<td>TBAA</td>
</tr>
<tr>
<td>Tripropylammonium</td>
<td>Acetate</td>
<td>TPAA</td>
</tr>
<tr>
<td>Hexylammonium</td>
<td>Acetate</td>
<td>HAA</td>
</tr>
<tr>
<td>Triethylammonium</td>
<td>Hexafluoroisopropanol</td>
<td>TEA-HFIP</td>
</tr>
</tbody>
</table>

- Selectivity and resolution impacted by choice
- TEA-HFIP remains the most effective for single-stranded oligo analysis in terms of LC-MS sensitivity and resolution, and is used throughout this presentation
TEAA vs. TEA-HFIP for IP-RP Separation of an Oligo Mixture

Triethylammonium Acetate Reagent

Triethylammonium HFIP Reagent
Recent work: Alternative HFIP LC/MS Buffer systems

- **Triethylamine**
  - 400 mM HFIP, pH 8.0
  - Cost: $$$$$
  - "standard"

- **Butylamine**
  - 50 mM HFIP, pH 9.0
  - Cost: $$$
  - 10x less

- **Dibutylamine**
  - 25 mM HFIP, pH 9.5
  - Cost: $
  - 20x less
BEH Columns Perform well under High pH

**15mM BA:50mM HFIP, pH 9.0**

BEH, C<sub>18</sub>, 135Å, 1.7µm

50 x 2.1 mm column

Stable chromatography over 400 injections
Ruggedness of BEH Columns

**Hour 29, inj # 53**

- 15nt
- 20nt
- 25nt
- 30nt
- 35nt

**Hour 124, inj # 243**

**Hour 193, inj # 421**

---

**Average Peak width ($W_{50}$) vs. Hours at pH 9.0**

*stable column performance in harsh conditions*

<table>
<thead>
<tr>
<th>Peak Width ($W_{50}$)</th>
<th>Inter assay</th>
<th>Intra assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>&lt; 0.08 min</td>
<td>0.08 min</td>
</tr>
<tr>
<td>S.D.</td>
<td>&lt; 0.003</td>
<td>0.002</td>
</tr>
<tr>
<td>R.S.D (%)</td>
<td>&lt; 3.5</td>
<td>2.49</td>
</tr>
</tbody>
</table>

Highly Reproducible and robust column performance over 200 hours at pH 9.0, 60 °C using butylamine/ 50mM HFIP buffer
Troubleshooting Oligonucleotide LC-MS
- Quality of Mobile Phase Matters!

**LC-MS grade solvents and additives should be used**

MS-grade TEA purchased from Sigma (Fluka 65897-50ML-F)

MS-grade HFIP purchased from Sigma (Fluka 42060-50ML)
Troubleshooting Oligonucleotide LC-MS
- Mobile Phases Need to be Fresh!

We recommend mobile phases be prepared daily to prevent MS signal loss.
Troubleshooting - System Cleanliness Matters!

Systems previously used for aqueous based separations may require a multi-step cleaning protocol. LC-HRMS/HDMS systems will require extended flushing with 50:50 H₂O:MeOH, 0.1%FA to reduce phosphate adduct from cleaning procedure.
An effective protocol for reducing metal adducts

**Reduction of metal adducts in oligonucleotide mass spectra in ion-pair reversed-phase chromatography/ mass spectrometry analysis**


Describes an intermittent wash strategy that prevents the accumulation of metal adducts over time, which can cause MS signal degradation and inconsistent performance.
Contains 24 application notes that demonstrate the oligonucleotide separations performance and productivity that can be achieved using Waters column chemistries, reagent standards, UPLC systems, and LC-MS workflows:

- UPLC-MS
- UPLC-HRMS
- UPLC-HDMS

Download @ waters.com/oligos
UPLC-MS

Screening for Oligo ID and Purity with the ACQUITY QDa Mass Detector

Our most deployable and scalable solution
Easy-to-use with a low maintenance requirement
Quickly confirm product ID & assess purity
The ACQUITY QDa Detector

- Empowering analytical chemists and chromatographers everywhere with orthogonal mass detection - see what lies under every peak

- Innovative compact design focused on ease-of-use, robustness and reliable performance

- Seamlessly integrates into Empower CDS for deployment in regulated & non-regulated labs
As Easy to Deploy as an Optical Detector

- Empower® and MassLynx™ Control
- Minimal operator training required
- Qualification Service available
- 110/220V operation – no need for special outlet
- Workhorse with low maintenance requirement
Automated Start Up and Calibration

- **Resolution and calibration** verified automatically with each start-up ensures mass data is accurate and reproducible. **No tuning required!**
- **ESI source optimized** for UPLC/UHPLC

**Graphic QDa monitor enables easy viewing and adjustment of system parameters**
Familiar User Interfaces for Ease-Of-Use and Deployment

Interface just like that of a PDA
- Minimal training needed
- Quick addendum to current SOP

Brings added MS functionality
- Ability to deconvolute data
- Export to ProMass for assignments
Disposable Components for Easy Maintenance

Sample Aperture:
As easy to replace as a detector lamp

Capillary:
Pre-assembled capillary - no cutting or assembly required
A Tool for Development and Production

- Greater insight into every peak
- Accelerated method and process development
- More sensitive and selective attribute monitoring
- Streamlined workflows for improved productivity
- Compact, robust, easy-to-use and ready to deploy
QDa Proof-of-Principle with MassPREP Standard

green = observable charge states

- Multiple charge states observed per oligonucleotide
- Detection readily observed with low pmol column loads
- Quantification based on UV Channel with MS providing mass confirmation

<table>
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<tr>
<th>nt</th>
<th>Avg. MW</th>
<th>[M-4H]</th>
<th>[M-5H]</th>
<th>[M-6H]</th>
<th>[M-7H]</th>
<th>[M-8H]</th>
<th>[M-9H]</th>
<th>[M-10H]</th>
<th>[M-11H]</th>
<th>[M-12H]</th>
<th>[M-13H]</th>
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<th>[M-15H]</th>
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<td>662.0</td>
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<td>1511.1</td>
<td>1327.1</td>
<td>1175.1</td>
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<td>813.2</td>
<td>755.0</td>
<td>704.6</td>
<td>660.5</td>
<td>621.6</td>
</tr>
</tbody>
</table>

P/N 186004135
Robust Reproducible Performance

![TIC Trace: 50 pmol on-column](image)

<table>
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<tr>
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<th></th>
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<th></th>
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<tr>
<td>Expected m/z</td>
<td>1132.0</td>
<td>1006.1</td>
<td>905.4</td>
<td>823.0</td>
<td>754.3</td>
<td>696.2</td>
<td>646.4</td>
<td>603.2</td>
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<tr>
<td>Average m/z</td>
<td>1132.1</td>
<td>1006.1</td>
<td>905.4</td>
<td>823.1</td>
<td>754.3</td>
<td>696.2</td>
<td>646.5</td>
<td>603.4</td>
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<tr>
<td>Delta</td>
<td>+0.1</td>
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<td>0.0</td>
<td>+0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>+0.1</td>
<td>+0.2</td>
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<td>%RSD</td>
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<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
</tr>
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</table>

All data within QDa mass accuracy specification: +/- 0.2
Manual Deconvolution with MaxEnt1

Calculated Average Mass of 30nt = 9,063.8 Da

MaxEnt1 deconvolution available within MassLynx provides for manual deconvolution of nominal mass and high resolution data.
Enabling Automated Data Workflows with ProMass

- "Automated" deconvolution and reporting package
- Nominal mass and high resolution mass spec (HRMS) data
- Enables higher throughput workflows

+ Znova™ deconvolution for nominal mass data (Quadrupole)
+ Respect™ deconvolution for high resolution data (QTof) – available with Promass HR
# ProMass Workflow

<table>
<thead>
<tr>
<th>MassLynx Acquisition</th>
<th>ProMass “Bridge”</th>
<th>ProMass Processing and Reporting</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Acquisition of LC-MS(/{MS}) data</td>
<td>• Transfers data from MassLynx to ProMass for automated batch processing / reporting</td>
<td>• Automated deconvolution</td>
</tr>
<tr>
<td>• Automatically calls ProMass Bridge</td>
<td></td>
<td>• Assess / confirm target mass and purity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Impurity peak assignments</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Report Generation</td>
</tr>
</tbody>
</table>
ProMass: Deconvolution Parameters

- **Peak width** set to span the width at the base of most peaks from the ESI mass spectra. A typical Peak Width value is 2-3 m/z units.

- **Merge Width** defines the m/z window were intensities are summed (merged) to compute a centroided value. Typically 10-25% of the Peak Width (e.g., 0.2-0.5 for 2 m/z unit peaks)
ProMass: Batch Processing Parameters

Results Tab

Reporting Tab
ProMass: Report Generation

**Summary Report**

- **Deconvolution Peak Reports**
- **Deconvoluted Spectrum**
- **Magnified Deconvolution ESI Mass Spectrum**

• A top-level summary report shows results from multiple runs and provides hyperlinks that allows users to drill down into sample-specific results

• Target mass features allows one to automatically search for one or more target masses from the LC-MS data

**Note:**

- Requires dongle to process (can not remote in)
ProMass: ZNova Mass Accuracy

### Table: Mass Accuracy

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<thead>
<tr>
<th>n=3</th>
<th>nt 15</th>
<th>nt 20</th>
<th>nt 25</th>
<th>nt 30</th>
<th>nt 35</th>
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<tbody>
<tr>
<td>Expected Mass (Da)</td>
<td>4,500.9</td>
<td>6,021.9</td>
<td>7,542.9</td>
<td>9,063.8</td>
<td>10,584.8</td>
</tr>
<tr>
<td>Observed Mass (Da)</td>
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<td>6,022.0</td>
<td>7,543.3</td>
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<td>10,585.3</td>
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<tr>
<td>Δ mass</td>
<td>-0.1</td>
<td>0.1</td>
<td>0.4</td>
<td>0.0</td>
<td>0.5</td>
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<tr>
<td>SD</td>
<td>0.06</td>
<td>0.17</td>
<td>0.20</td>
<td>0.10</td>
<td>0.06</td>
</tr>
</tbody>
</table>

- 2 min window centered around each peak used for combined spectra
- QDa mass data deconvoluted with Promass Znova™ algorithm
- Mass errors within 1.0 Da of expected
QDa Enabled Mass Confirmation & Impurity Profiling

**Sample:** Two distinct 21nt ssRNA oligos representing the complementary upper and lower strands of a siRNA duplex (10 pmol/uL)

**Upper Strand,** MW 6693.1 Da 5’-UCGUCAAGCGAUUACAAGGTT-3’

**Lower Strand,** MW 6607.0 Da 5’-TTCCUUGUAUAUCGCUUGACGA-3’

**4 Minute Gradient:** 0.5%/min

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (ml/min)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0.200</td>
<td>82.0</td>
<td>18.0</td>
<td>0.0</td>
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<tr>
<td>4.00</td>
<td>0.200</td>
<td>80.0</td>
<td>20.0</td>
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<tr>
<td>4.01</td>
<td>0.200</td>
<td>50.0</td>
<td>50.0</td>
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<tr>
<td>6.00</td>
<td>0.200</td>
<td>50.0</td>
<td>50.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>6.01</td>
<td>0.200</td>
<td>82.0</td>
<td>18.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

TUV, 260 nm

**Upper Strand (50 pmol)**

QDa, 410-1250 m/z
Summary Report - Batch Results with Interactive Display

Target Mass: 6693.1 Da (Upper Strand)

- Data for 48 samples processed in < 10 min
- 100% match with expected results - displayed in easy-to-use color-coded format
- Multiple target masses can be searched
- 96-well plate format
Interactive Sample Report

Total Ion Chromatogram

Raw ESI Spectrum

Deconvoluted Spectrum

Blue Text = Hyperlinks to data views
Retention Time and Mass Accuracy

Consistent retention times across all 42 samples

All data within 1 Dalton of expected mass
ProMass: Spectral Analysis for Purity

Non target masses other than predicted adducts are calculated as impurities
Oligonucleotides – Detecting Modified Oligos

A) siRNA unmodified

B) Fully Thioated (PS) DNA

C) 2’Ome Modified DNA

Mass information acquired with the QDa used to confirm product identity based on average mass with excellent agreement between theoretical and experimental MW.
UPLC-HRMS
Characterization and HRMS Monitoring
with the Xevo G2-XS Qtof

Robust High Resolution MS
Enhanced Sensitivity for Characterization Work
MS/MS for Sequence Confirmation
High Res Performance with MassPREP Standard

**Instrumentation**
- LC: Waters ACQUITY® H-Class Bio
- MS: Xevo G2-XS Qtof

**Column Chemistry**
- ACQUITY UPLC OST BEH C18 Column 130Å, 1.7 µm, 2.1 mm X 50 mm (P/N: 186003949)

**Waters MassPREP Oligonucleotide Standard**
- Reconstituted to 5 pmol/µL in pure H2O
- 1 µL of sample injected /LC-MS analysis

**Method**
- 15 min Ion Pairing RP gradient (TEA/HFIP)
- Data collected by TUV and by ESI- MS
MassPREP Standard: TUV and TIC comparison

Load: **5 pmol** on-column

**TUV**

**TIC**
MassPREP Standard - Raw ESI Spectra for 25nt Oligo

5 pmol on-column

M<sup>3-</sup> charge state isotopic distribution

Resolution: > 30K

+Na<sup>+</sup>

m/z 2508 - 2530

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Deconvoluted spectrum for 30nt

<table>
<thead>
<tr>
<th>OST Standard</th>
<th>Expected Mass</th>
<th>Observed Mass</th>
<th>Mass Accuracy (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15nt</td>
<td>4498.7348</td>
<td>4498.7300</td>
<td>-1.07</td>
</tr>
<tr>
<td>20nt</td>
<td>6018.9650</td>
<td>6018.9730</td>
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<tr>
<td>25nt</td>
<td>7539.1952</td>
<td>7539.1990</td>
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</tr>
<tr>
<td>30nt</td>
<td>9059.4254</td>
<td>9059.4210</td>
<td>-0.49</td>
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<tr>
<td>35nt</td>
<td>10579.656</td>
<td>10579.6260</td>
<td>-2.79</td>
</tr>
</tbody>
</table>

Avg. 1.24

Mass accuracy: < 5 ppm

Promass HR includes the Respect™ deconvolution algorithm for processing high res MS data
MassPREP Standard - Determining Limit of Detection (LOD)

Load: **20 fmol** on-column

Detection limited by UV signal, not MS sensitivity
Evaluating performance with ssRNA strands

**Sample:** Two distinct 21nt ssRNA oligos representing the complementary upper and lower strands of a siRNA duplex (10 pmol/μL). 10 minute gradient for high resolution chromatogram

**Upper Strand,** MW 6693.1 Da
5’-UCGUCAAGCGAUUAACAAGGTT-3’

**Lower Strand,** MW 6607.0 Da
5’-TTCCUUGUAAUCGUUGACGA-3’

1 μL injected for LC-MS analysis.

Oligo sourced from IDT
A ssRNA sequence 5’-UCGUCAAGCGAUUACAAGGTT-3’ with a double thymine overhang was separated from the base deletion (N-1) and base insertion (N+1) forms using a 10 min high resolution separation gradient from 13% B to 21% B.

\[
\begin{align*}
N-1 &= 5’-\text{rUrCrGrUrCrArArGrGrUrUrArCrArArGrGrTT-3’} \\
\text{Average mass} &= 6386.9 \text{ Da; ProMass HR observed mass:6386.8 Da} \\
N &= 5’-\text{rUrCrGrUrCrArArGrGrUrUrArCrArArGrGrTT-3’} \\
\text{Average mass} &= 6693.1 \text{ Da; ProMass HR observed mass:6693.0 Da} \\
N+1 &= 5’-\text{rGrUrCrGrUrCrArArGrGrUrUrArCrArArGrGrTT-3’} \\
\text{Average mass} &= 7038.3 \text{ Da; ProMass HR observed mass:7038.7 Da}
\end{align*}
\]
Extracted Ion Chromatograms (XIC) of N-1, N and N+1
Upper Strand: QTof ESI Raw Spectrum

M³⁻ charge state isotopic distribution.
Summary Report of Batch Results with Interactive Display

- **Promass HR Respect™ algorithm used**

- **Target Mass: 6689.9550 Da (Upper Strand)**

- **Data processed for all 48 samples in < 10 minutes**

- **100% match with expected results; displayed in easy-to-use color-coded format**

### Promass Sample Browser

<table>
<thead>
<tr>
<th>File Name</th>
<th>Vial</th>
<th>Sample ID</th>
<th>Sequence</th>
<th>Target Masses</th>
<th>Observed Masses</th>
<th>Purity</th>
<th>Result Code</th>
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</table>
Interactive Sample-Specific Reporting

Total Ion Chromatogram

Raw ESI Spectrum

Deconvoluted Spectrum

Blue Text = Hyperlinks to Data Views
Collision of oligonucleotides with gas molecules at elevated energies produces characteristic fragment ions at each residue.

McLuckey Fragmentation Scheme

$c$, $y$, & $w$ ions predominate with QTof fragmentation.
LC-MS/MS of m/z = 1650 (-4 Peak) Deconvoluted Spectrum

Most C, Y, & W ions are matched
LC-MS/MS Sequence Confirmation - Upper Strand

5 pmol on-column

5’-U C G U C A A G C G A U U A C A A G G T T -3’

*Deconvolution using MaxEnt-3
UPLC-HDMS

Characterization with Ion Mobility using the SYNAPT G2-Si QTof

Ion Mobility brings an added dimension of separation that provides greater spectral clarity for enhanced selectivity and sensitivity, especially for low level impurities, and it enables 3D structure analysis.
Techniques that can provide enhanced sensitivity and selectivity

- **Charge State / Drift Time Stripping**
  - Reduces spectral complexity; makes data more suitable for deconvolution
  - Observe fragment ion series for each IMS resolved charge state

- **Wideband Enhancement**
  - Improve the resulting signal intensity (ca. 5-10 fold) of MS/MS spectra for low level impurities

- **Time Aligned Parallel (TAP) Fragmentation**
  - Separation of primary fragment ions via ion mobility enables selection of specific primary fragment ions for secondary fragmentation - Pseudo MS$^3$
Analysis of PEG 4450 via HDMS
DriftScope™ data showing the gas-phase separation power of the SYNAPT HDMS System. Components with different charge states are separated via ion mobility.

Mass spectrum showing the ions with +2 charge state. Improved mass spectral clarity makes data more suitable for charge state deconvolution algorithms.

Waters Application Note: Characterizing Polyethylene Glycol (PEG) by SYNAPT High Definition Mass Spectrometry. Weibin Chen et.al.
Wideband Enhancement

Fragmentation in Trap

Mobility separation

Precursor Ion Selected when ion current exceeds user threshold

Tof Pusher synchronized with series of ions in WB Enhancement
Time Aligned Parallel (TAP) Fragmentation

- Precursor ion
- Primary Fragments (Trap)
- Secondary Fragments (Transfer)

1° Fragment Ions IMS Separated
1° Fragment Ions & 2° Product Ions Drift Time Aligned
## In Summary: LC-MS Workflows for Oligonucleotide Analysis

<table>
<thead>
<tr>
<th>Feature</th>
<th>UPLC-MS ACQUITY QDa</th>
<th>HPLC-HRMS Xevo G2-XS QTof</th>
<th>UPLC-HDMS SYNAPT G2 Si QTof</th>
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<tbody>
<tr>
<td>Mass Accuracy – Deconvoluted Data</td>
<td>+/- 1.0 Dalton</td>
<td>&lt; 5 PPM (~0.038 Da)</td>
<td>&lt; 5 PPM (~0.038 Da)</td>
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<tr>
<td>Typical Column Load</td>
<td>50 pmol</td>
<td>5-10 pmol</td>
<td>5-10 pmol</td>
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<tr>
<td>Limit of Detection</td>
<td>&lt; 10 pmol</td>
<td>20 fmol</td>
<td>20 fmol</td>
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<td>Mass Confirmation</td>
<td>★★★</td>
<td>★★★★</td>
<td>★★★★</td>
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<tr>
<td>Impurity Profile</td>
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<td>MS/MS Sequencing</td>
<td>★ (in source)</td>
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<td>Cost</td>
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</table>
Appendix 1
Bio Accord Intact Mass Analysis Enhancement
New Target Mass Screening / Mass Confirmation Workflow
Who Needs Compliance-Ready Mass Confirmation?

- **Oligonucleotide Synthesis Houses**
  - Confirm identity and purity of therapeutic modalities

- **DNA based Diagnostic Test Kit Providers**
  - Confirm identity and purity of primers and probes

- **DNA Barcoding Companies**
  - Confirm identity of oligo-barcodes

- **Therapeutic Developers**
  - Incoming QC inspection on outsourced oligos
New Target Mass Screening / Mass Confirmation Workflow

- Compliance-ready data acquisition, processing and reporting
- Target mass screening via Intact Mass Analysis workflow
  - Sequence entry not required, just target mass(es) of oligonucleotides, proteins and related impurities
    - Peptides handled via Accurate Mass Screening Workflow – no deconvolution
  - New negative ion data processing enables oligo data analysis/mass confirmation
    - Deconvolution available with BayeSpray or MaxEnt1
Compliance-Ready Mass Confirmation of Oligonucleotides
Enter Target Mass(es) into Component Table

<table>
<thead>
<tr>
<th>Component name</th>
<th>Expected RT (min)</th>
<th>Time window (min)</th>
<th>Expected mass (Da)</th>
<th>Formula</th>
<th>Description</th>
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<tbody>
<tr>
<td>1 0.715</td>
<td>3.94</td>
<td>0.3</td>
<td>4500.9581</td>
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<td>2 0.720</td>
<td>6.66</td>
<td>0.3</td>
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<td>3 0.725</td>
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<td>10584.7967</td>
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</table>

Select Deconvolution Algorithm:

Select Molecule Type & Parameters:

MassPrep OST Oligo Standard P/N 186004135

New Choices
Processing Results for MassPrep OST Oligos

Average mass accuracy error: 4.7 ppm

Compliance-ready mass confirmation of oligos with very good mass accuracy
### Component Table for a 25mer Phosphothioated (PS) Oligo

<table>
<thead>
<tr>
<th>Component name</th>
<th>Expected RT (min)</th>
<th>Time window (min)</th>
<th>Expected mass (Da)</th>
<th>Formula</th>
<th>Description</th>
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<td>25-mer PPT oligo</td>
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</table>

**Fully Phosphothioated 25mer Oligo**

New isotopic model for deconvolution; enhances mass accuracy when working with phosphothioated species.
Processing results for a 25-mer PPT oligo

Mass accuracy error: 6.7 ppm
Compliance-Ready Mass Confirmation of Proteins
Component Table for three mAb’s

Enter masses for each mAb (target mass) into the component table.

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<thead>
<tr>
<th>Component name</th>
<th>Label</th>
<th>Item tags</th>
<th>Expected RT (min)</th>
<th>Time window (min)</th>
<th>Expected mass (Da)</th>
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<td>mAb2</td>
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<td>mAb3</td>
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<td>2.30</td>
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<td>148515.0</td>
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</table>

Select molecule type:
Review Pane: 1. Injection heat map
- (Sample A2) 2. Sample list
3. Identified components
4. TIC and TUV traces
5. Raw & Deconvoluted spectra

- TIC
- Raw Spectra
- Deconvoluted Spectra
- TUV
- Theoretical (Mock) Spectra
- Centroid
In Summary

- With UNIFI version 1.9.9, a new Target Mass Screening capability is now available within the Intact Mass Analysis workflow of the BioAccord.
- This capability enables Compliance-Ready mass confirmation of Oligonucleotides, Proteins and Impurities.
- All data acquisition, processing and reporting is done within UNIFI software, which offers user level security, secure data storage, and a complete audit trail capability so customers can ensure data integrity when providing QC reports or submitting regulatory filings.
Thank You

To Learn More Visit: [WWW.Waters.com/oligos](http://WWW.Waters.com/oligos)

or Contact your local Waters Account Representative