

## 100 Samples per Hour: High-Speed Oligonucleotide Analysis with BioAccord™ LC-MS and INTACT Mass Application

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*Published on November 27, 2025*

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### Abstract

The development of oligonucleotide therapeutics requires fast, reliable analytical workflows to support screening, synthesis, and quality control. This application note presents a streamlined solution using the Waters BioAccord LC-MS System operated under the waters\_connect™ Informatics Platform, utilizing the INTACT Mass Application to deliver automated mass confirmation and purity determination of oligonucleotides. By integrating robust LC-MS capabilities with intelligent and automated data processing, the system enables acquisition and analysis of over 100 samples per hour. The automated workflow minimizes manual interventions, enhances the consistency of results and enables scientists to make faster, more informed decisions in early-stage development.

### Benefits

Whether screening early candidates or assaying batches of a commercial product, the BioAccord LC-MS System combined with the waters\_connect INTACT Mass App (Figure 1) delivers speed, automation, and precision.

1. Fast, scalable, and automated

- This study demonstrates the analysis of over 100 samples per hour with automated workflow integration from acquisition to processed results.
- The automated processing eliminated manual steps associated with defining parameters for peak detection, mass spectral deconvolution, impurity identification, and report generation.

## 2. Precision that delivers

- Increased deconvoluted mass accuracy was achieved using the chemical formula for each sample to improve the deconvolution model.
- Embedded purity calculations processed optical chromatograms, TICs, or mass spectra, within the INTACT Mass Application to deliver consistent results.
- Targeted impurities were identified, reporting delta masses for novel, non-targeted species, simplifying downstream investigations.

## 3. Intuitive insights and visualization

- Color-coded dashboards for mass confirmation and purity thresholds simplified sample review, an important element for eliminating a key bottleneck in high throughput analysis.
- The software flags exceptions to the expected results, with grouped failed samples presented for efficient review.

## 4. Flexible platform, compliance-ready

- The platform enables rapid deployment of validated methods with a single vendor, compliance-ready software environment for data acquisition, data processing, and result reporting.



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*Figure 1. The BioAccord LC-MS System with ACQUITY™ Premier UPLC™ System, comprised of an ACQUITY Premier Binary UPLC, Tunable UV Detector (TUV) coupled in-line with ACQUITY RDa™ Mass Detector, operated under the waters\_connect Informatics Platform.*

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## Introduction

Synthetic oligonucleotides are increasingly recognized as versatile therapeutic agents, offering high specificity and the ability to target previously inaccessible biological pathways. As the scope of oligonucleotide-based therapies expands from highly modified antisense oligonucleotides (ASOs) to siRNAs and sgRNAs, so does the need for robust analytical workflows capable of handling diverse chemistries, modifications, and sample complexity.

Unlike protein-based biotherapeutics, most oligonucleotides are produced via chemical synthesis, allowing for

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precise control over sequence and structure. However, even with highly optimized synthetic protocols, it remains essential to confirm molecular mass, assess purity, and identify potential failure sequences or synthetic impurities.

This application note presents a higher throughput LC-MS workflow using the Waters BioAccord LC-MS System and INTACT Mass App on the waters\_connect Platform, with a workflow designed to automate the routine analysis of synthetic oligonucleotides. The data confirmed successful analysis of oligonucleotides ranging from 10-mer to 60-mer, including ASOs with diverse backbone and sugar modifications. While this range reflects typical therapeutic oligonucleotides, the software can support even greater sequence lengths and structural diversity.

Experimental results demonstrated the system's ability to deliver rapid, accurate mass confirmation and impurity profiling across a wide oligonucleotide landscape. With acquisition and processing times under 30 seconds per sample, throughput can exceed 100 samples per hour. This facilitates scalable, reliable analysis for screening, development, and quality control of oligonucleotides, empowering researchers to make confident decisions at every stage of analysis.

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## Experimental

A reversed-phase trap and elute LC-MS method was developed to enable high-throughput intact mass confirmation screening of synthetic oligonucleotides. To demonstrate the method's versatility, a range of Waters MassPREP™ Oligonucleotide Standards with concentrations ranging from 0.1 mg/ml to 0.01 mg/ml were analyzed alongside representative antisense oligonucleotides (ASOs), including GEM-91 and nusinersen. These examples encompass a diverse array of oligonucleotide chemistries, lengths, and structural complexities. The study highlights the capability of the workflow to acquire and process data for diverse oligonucleotide samples in a single batch, using a single method, and delivering rapid and reliable mass confirmation and impurity results.

## LC Conditions

LC system:	Waters ACQUITY Premier UPLC System (Binary)
Detection:	UV 260 nm

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Vials:	TruView™ LCMS Certified 12 x 32 mm Screw Neck Vial, Total Recovery, with Cap and Preslit PTFE/Silicone Septum (p/n:186005663CV)
Column:	ACQUITY Premier Oligonucleotide C <sub>18</sub> VanGuard™ FIT Cartridge, 130 Å, 1.7 µm 2.1 mm x 5 mm (p/n:186010696) with VanGuard Cartridge Holder (p/n:186007949)
Sample temperature:	6 °C
Injection volume:	1 µL
Flow rate:	1.00 mL/min
Mobile phase A1:	7 mM triethylamine (TEA) and 40 mM hexafluoro-2-propanol (IonHance™ HFIP Additive (p/n:186010781) in Milli-Q® water (pH 8.6)
Mobile phase B1:	3.5 mM TEA, 20 mM HFIP in 50% methanol

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	1.00	80	20	Initial
0.1	1.00	40	60	11
0.2	1.00	80	20	11
0.35	1.00	80	20	6

## MS Conditions

MS system:	Waters BioAccord LC-MS System
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Mode:	LC-MS <sup>E</sup>
Mass range:	400–5000 <i>m/z</i>
Polarity:	Negative
Scan rate:	10 Hz
Cone voltage:	40 V
Source temperature:	120 °C
Desolvation temperature:	550 °C
Capillary voltage:	0.8 kV
MSE collision energy ramp:	70–110 eV
Lockmass correction:	Scheduled lockmass

The “scheduled lockmass” option was selected in the MS method for this experiment to shorten the total acquisition time; by reducing inter-sample system check activities from a per-injection basis to once every 60 minutes. With the “scheduled lockmass” selected in the INTACT Mass Application, it required only 25 minutes to complete LC-MS data acquisition and data processing for a 48-well tray of oligonucleotide samples.

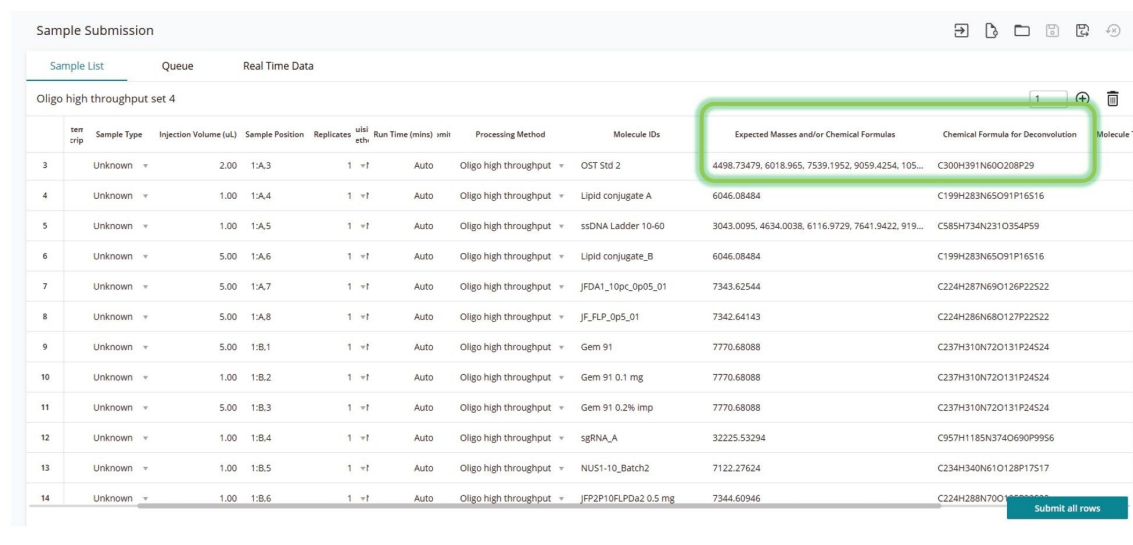
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## Results and Discussion

High-throughput analyses present subtle challenges beyond the needs of evaluating a single sample within a batch. Software supporting higher throughput must be flexible enough to deal with analyte diversity within a batch without making methods unnecessarily complex. To address these analytical challenges, the waters\_connect INTACT Mass App version 1.9, combined with enhancements to the Sample Submission App

2.7.0, simplifies higher throughput analyses by allowing users to specify either expected masses or chemical formulas directly within the sample list (Figure 2). This significantly simplifies setup, rather than requiring individual methods for each analyte. Additionally, specifying chemical formulas for target molecule assignments enhances the deconvolution model, improving mass accuracy during charge deconvolution. In this study, specifying target chemical formulas enabled successful batch analysis of a diverse range of oligonucleotide samples using a single acquire-and-process method. This approach enabled processing of chemically varied analytes within the same analysis, delivering optimized results for each sample, without the need for manual intervention.

In this study, the BayesSpray deconvolution algorithm with monoisotopic result outputs was used to deliver high precision results for the analyzed oligonucleotide samples.



Sample Submission											
Sample List		Queue	Real Time Data								
Oligo high throughput set 4											
ten trip	Sample Type	Injection Volume (uL)	Sample Position	Replicates	initial event	Run Time (mins)	smil	Processing Method	Molecule IDs	Expected Masses and/or Chemical Formulas	Chemical Formula for Deconvolution
3	Unknown	2.00	1:A,3	1	✓f	Auto	Oligo high throughput	OST Std 2		4498.73479, 6018.965, 7539.1952, 9059.4254, 105...	C300H391N60O208P29
4	Unknown	1.00	1:A,4	1	✓f	Auto	Oligo high throughput	Lipid conjugate A		6046.08484	C199H283N65O91P16516
5	Unknown	1.00	1:A,5	1	✓f	Auto	Oligo high throughput	ssDNA Ladder 10-60		3043.0095, 4634.0038, 6116.9729, 7641.9422, 919...	C585H734N231O354P59
6	Unknown	5.00	1:A,6	1	✓f	Auto	Oligo high throughput	Lipid conjugate_B		6046.08484	C199H283N65O91P16516
7	Unknown	5.00	1:A,7	1	✓f	Auto	Oligo high throughput	JFDA1_10pc_0p05_01		7343.62544	C224H287N69O126P22522
8	Unknown	5.00	1:A,8	1	✓f	Auto	Oligo high throughput	JF_FLP_0p5_01		7342.64143	C224H286N68O127P22522
9	Unknown	5.00	1:B,1	1	✓f	Auto	Oligo high throughput	Gem 91		7770.68088	C237H310N72O131P24524
10	Unknown	1.00	1:B,2	1	✓f	Auto	Oligo high throughput	Gem 91 0.1 mg		7770.68088	C237H310N72O131P24524
11	Unknown	5.00	1:B,3	1	✓f	Auto	Oligo high throughput	Gem 91 0.2% imp		7770.68088	C237H310N72O131P24524
12	Unknown	1.00	1:B,4	1	✓f	Auto	Oligo high throughput	sgRNA_A		32225.53294	C957H1185N374O690P9956
13	Unknown	1.00	1:B,5	1	✓f	Auto	Oligo high throughput	NUS1-10_Batch2		7122.27624	C234H340N61O128P17517
14	Unknown	1.00	1:B,6	1	✓f	Auto	Oligo high throughput	JFP2P10FLPDA2 0.5 mg		7344.60946	C224H288N70O127P22522

*Figure 2. Sample list setup enables targeted formula input for streamlined deconvolution and impurity analysis.*

When analyzing synthetic oligonucleotides, even in higher throughput screening, the analyst is often tasked with assessing critical product attributes beyond simple mass confirmation. This may include detection and reporting of impurities, including known adducts, n-1 modifications, and process-related impurities, recognizing mobile phase adducts as product-related species and reporting unexpected potential impurities by their delta mass from the target molecule.

Supporting this impurity logic within the automated data processing routines reduced the need for manual interventions and can accelerate decision-making, helping scientists move from raw data to actionable insights faster.

## Review and Reporting of Results

The dashboard view (Figure 3) provides a real-time summary of oligonucleotide injections during the acquire and process run. Color-coded flags offer instant feedback on each sample's status, confirming whether target masses were matched and purity thresholds met.

- Green: Pass — mass and purity within expected limits
- Orange: Warning — mass error or reduced purity
- Red: Fail — unmatched mass or purity below threshold

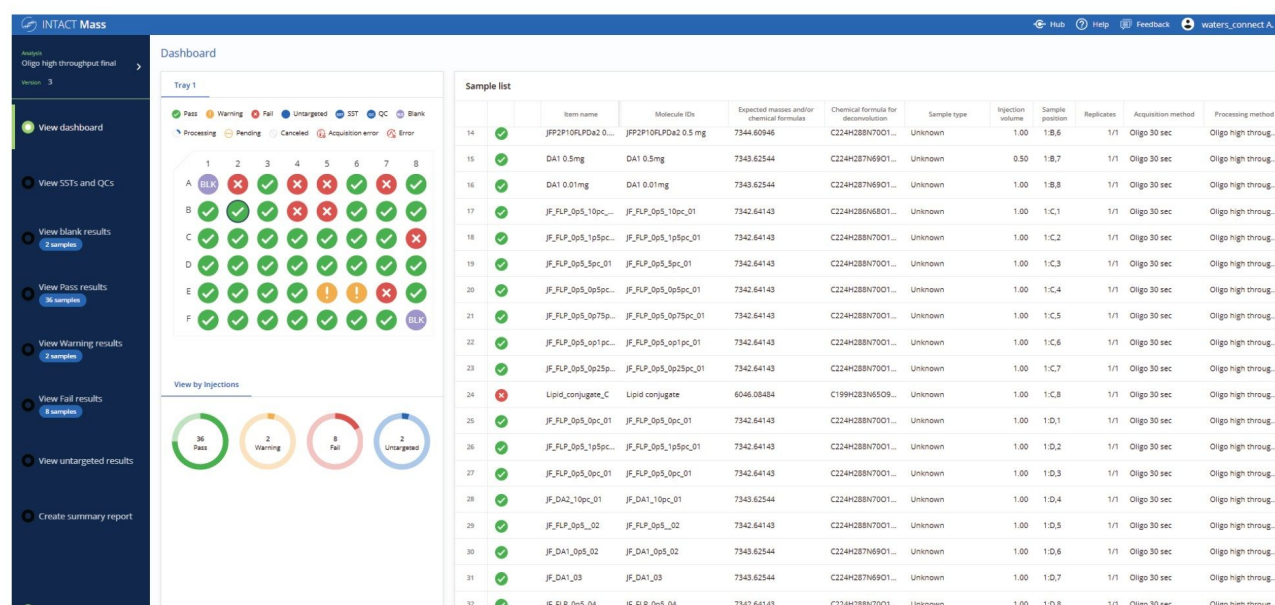


Figure 3. The dashboard results view. The color codes on the sample plate reflect the result status of the samples analyzed. The detailed individual sample results can be reviewed by clicking within the sample list.

Data review can be a bottleneck in high-throughput analysis, which can be overcome using graphical summaries and flags that quickly communicate the status of each analyzed sample. This is demonstrated in the



oligonucleotide plate analysis (Figure 3), where users can distinguish between samples that meet both target and purity criteria, those that fall short of acceptance thresholds, and those flagged for further review. The ability to bring up a grouped display of failed samples (Figure 4) allows the user to quickly focus their review even further and with more detailed results. Samples can be automatically flagged based on key criteria such as mass accuracy, absence of the target mass, or low calculated target purity. These exceptions are clearly visualized and grouped for efficient review, allowing users to quickly focus on problematic data and accelerate decision-making across large batches. In this example, although the target species was successfully identified, the sample was flagged due to not meeting the predefined purity threshold.

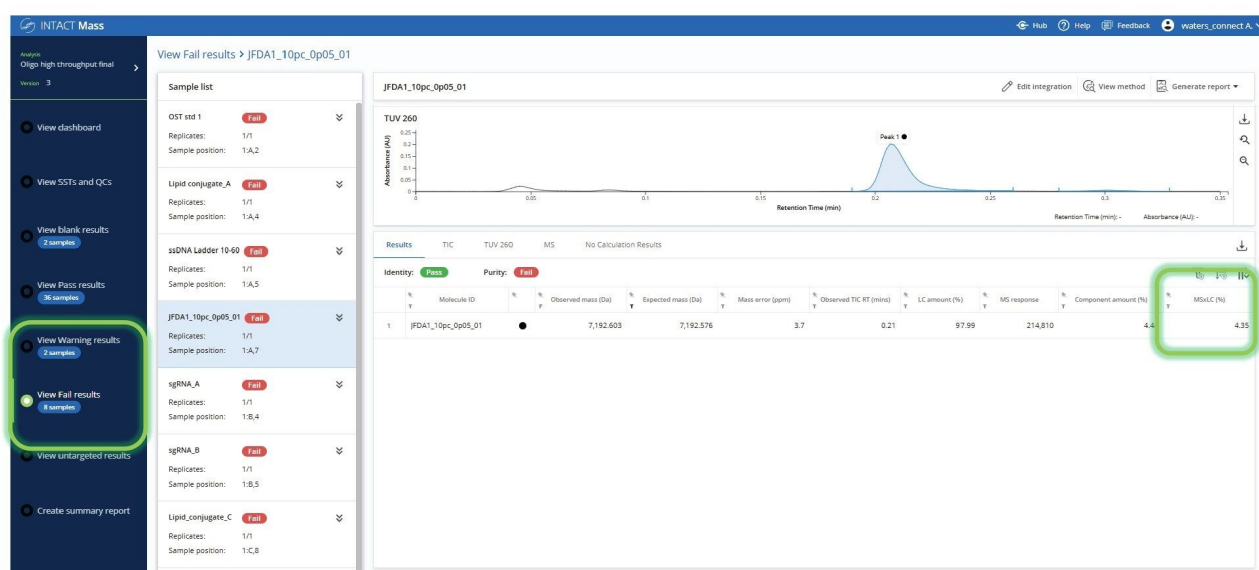
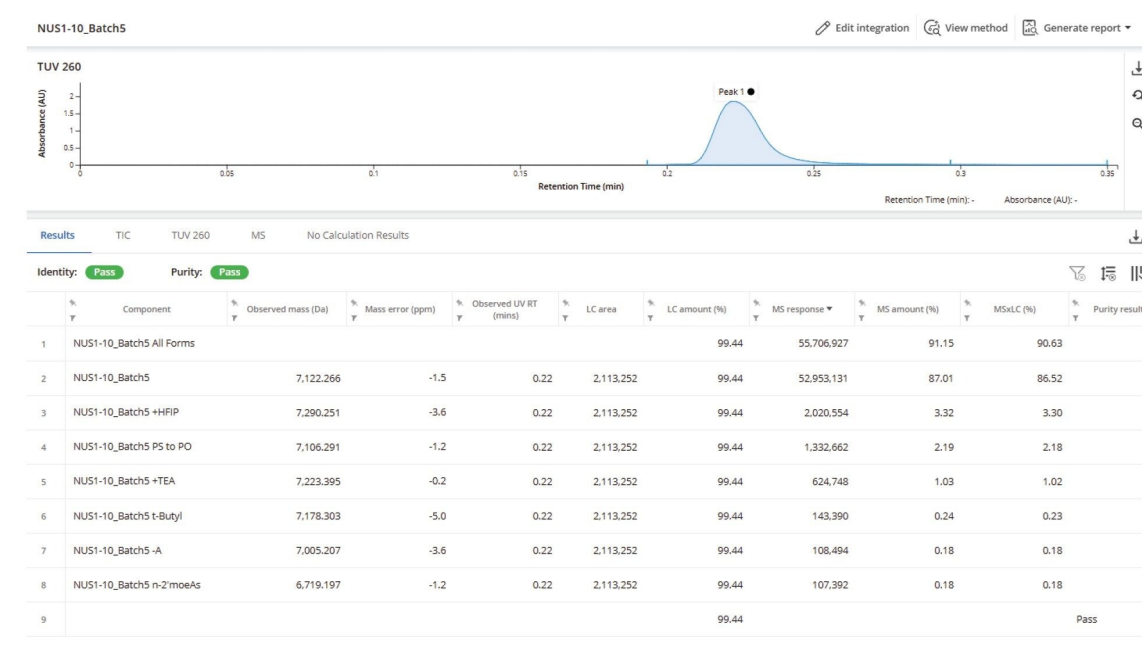


Figure 4. Efficient review of failed samples.

To gain deeper insights, results from individual samples can be reviewed individually. Several results spanning a diverse set of oligonucleotides acquired within this batch analysis are provided.

The first result features nusinersen, an oligonucleotide with a phosphorothioate backbone and 2'-O-methoxyethyl (MOE) sugar modifications. The elemental composition ( $C_{234}H_{340}N_{61}O_{128}P_{17}S_{17}$ ) differs from the typical default deconvolution model settings for oligonucleotides and PS oligonucleotides. By applying the chemical formula, a deconvoluted mass accuracy of -1.5 ppm was achieved for the main target species with no observed artifacts. A range of lower-level impurities and adducts were also identified, with the lowest impurity being quantified at

0.18%. (Figure 5). The INTACT Mass Application method enabled mobile phase adducts to be treated separately from impurities. These adducts can be incorporated into the overall purity percentage of the main product rather than quantified as distinct impurities.



*Figure 5. INTACT Mass App results for nusinersen. The INTACT Mass Application accurately deconvoluted the main species and identified low-level impurities and adducts, with the lowest impurity quantified at 0.18%.*

In a separate sample, GEM-91, a fully phosphorothioated 25-mer, was identified with high confidence, and several new impurities were reported. A delta mass calculation is applied to all MS peaks that were not targeted or otherwise identified. This hierarchy of automated assignments streamlines the characterization of novel impurities by highlighting the unexpected species present in the sample.

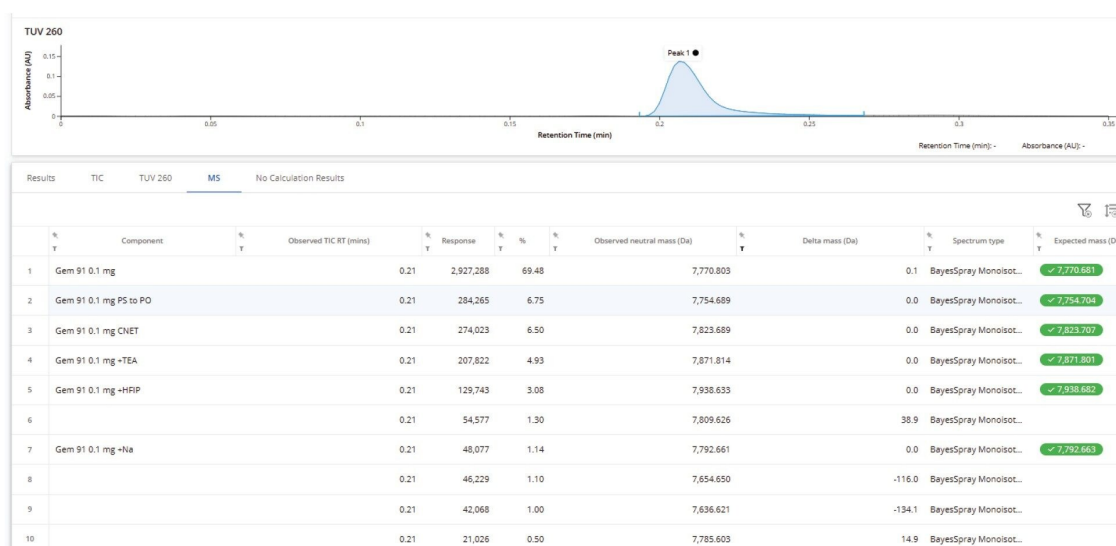


Figure 6. Analysis of GEM-91. The delta mass column (third from right) highlights unidentified MS peaks with mass differences relative to the expected target to simplify unknown impurity characterization.

A potential challenge when running batches of samples is the analysis of mixtures containing oligomer products of varying lengths and intensities. However, using the common method for this mixed batch, successful deconvolution and identification of all expected oligonucleotides in the Waters ssDNA ladder was demonstrated, which includes co-eluting species ranging from 10-mer to 60-mer. The lowest abundance species, 10-mer + HFIP, was identified with an MS×LC purity of 0.5% (Figure 7).

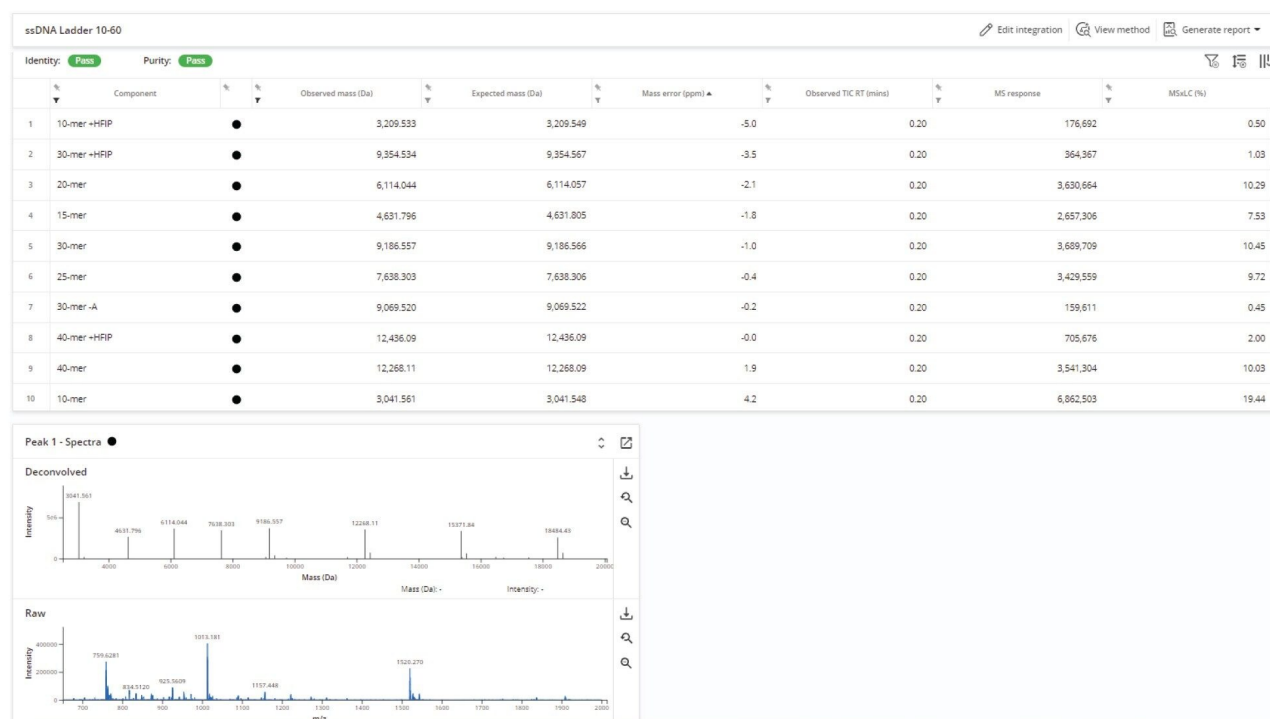


Figure 7. Automated identification of multiple oligonucleotides within a mixture from the resulting complex spectrum of the Waters ssDNA ladder, showcasing successful deconvolution of co-eluting of a ladder 10–60mer oligonucleotides.

## Reporting

Automated reporting ensures traceability and simplifies documentation for regulatory or internal review. When configuring the acquire and process method in the INTACT Mass App, users can opt to automatically generate a PDF report for each sample (Figure 8).

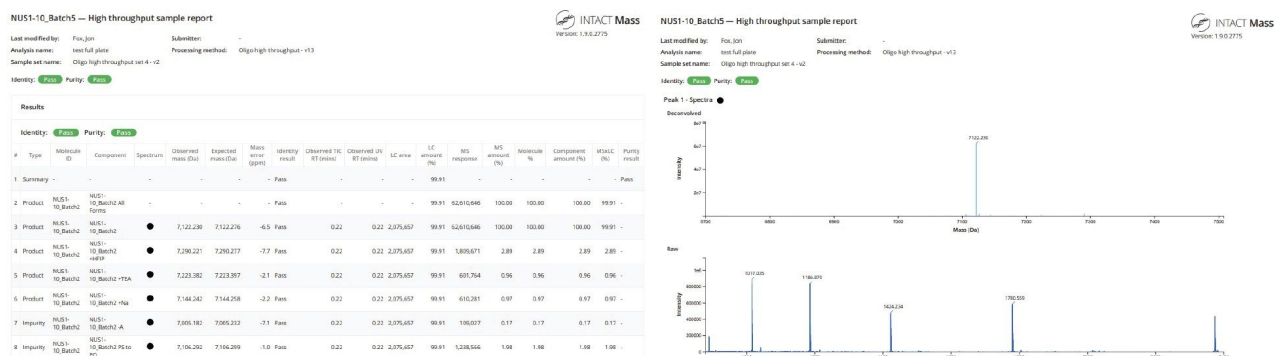


Figure 8. INTACT Mass App report. The report includes the experimental information, TIC, and TUV chromatograms with the integrated peaks noted (not shown), the identified components with their assigned modifications, and the calculated mass accuracy and purity information (left). The raw and deconvolved spectral data (right) are also visualized in the formatted report.

## Conclusion

The BioAccord LC-MS System with the waters\_connect INTACT Mass App provided a fast, automated solution for oligonucleotide screening analysis, with several key elements directly contributing to the efficiency and quality of higher throughput screening results:

- Rapid analysis: Over 100 oligonucleotide samples per hour throughput was achieved using a rapid LC-MS method, the scheduled lockmass setting for BioAccord System acquisition, and the Acquire and Process acquisition mode for sample to result automation.
- Automated data processing: Intelligent peak picking, mass spectral deconvolution parameter setting, and embedded impurity profiling logic enabled detailed insights on samples of varying complexity.
- Optimal mass spectral deconvolution: This can be achieved across a sample batch with diverse oligonucleotide types and lengths when using chemical compositions for targeted molecules to optimize the deconvolution model for each sample analyzed.
- Graphical batch summaries and review by exception logic: This enabled faster analyst review of data, and the

ability to more quickly gain insights on sample results that do not conform to quality criteria defined in the method.

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## References

1. Shion, H., Berger, S. J., Yu, Y. Q. (2020). Application of a Mass Confirmation Workflow for Biotherapeutics Screening. Waters Application Note. [720007027](#), November 2020
2. Shion, H., Boyce, P., Berger, S. J., Yu, Y. Q. (2022). INTACT Mass™ - a Versatile waters\_connect™ Application for Rapid Mass Confirmation and Purity Assessment of Biotherapeutics. Waters Application Note. [720007547](#), February 2022.

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720009127, November 2025



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