Application Note

Biopharma

Determination of Purity, Assay, and Impurity Profile for a Ligand-Conjugated Antisense Oligonucleotide

Using Agilent Oligo Analysis Accelerator for OpenLab CDS

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Abstract

This application note demonstrates how the Agilent Oligo Analysis Accelerator (OAA) for OpenLab CDS—an add-on for Agilent OpenLab CDS software—simplifies data analysis while leveraging an existing data integrity and compliance architecture. A phosphorothioate ligand-conjugated antisense oligonucleotide (LICA) was analyzed to determine its purity, assay, and impurity profile. With an optimized processing method, the total data analysis time was shown to be less than one hour per sample, depending on its complexity and purity.

Introduction

In therapeutic synthetic oligonucleotide development, there are several challenges associated with determining the sample purity, label claim, or assay, as well as quantitating impurities. This is of critical importance towards late-stage development and becomes a requirement to support oligonucleotide quality control (QC) and batch release. Traditional HPLC methods may not have the necessary sensitivity or specificity for QC testing. Using a more selective detector such as a mass spectrometer (MS) is a viable technique for QC testing needs. Indeed, the preferred approach is to quantitate high-level impurities using the UV signal, while identifying and quantitating lower-level impurities using the more selective and sensitive MS detection.

As demonstrated by Rentel et al., the Agilent InfinityLab LC/MSD XT can be used and validated for the QC lot release of oligonucleotides. This is due to the single quadrupole's ease of use, sensitivity in full-scan mode, and sufficient mass range to analyze longer, conjugated oligonucleotides. The LC/MS method uses both UV and MS signals to quantitate impurities. UV detection is used to quantify impurities that are separated chromatographically, while MS detection is used for impurities that coelute with the full-length product (FLP). Additionally, the analytical method used in this approach is unique in a couple of ways. Unlike most ion pair reversed-phase methods for oligonucleotides, the soft ionization conditions used focus the charge state distribution of the parent oligonucleotide and its impurities primarily into the –4 charge state. This simplifies the spectrum and increases sensitivity for the detection of low-level impurities. Further, to facilitate the differentiation of adducts of FLP from impurity peaks, the sample is injected under harsh source conditions, which removes peaks in the average mass spectrum due to buffer adducts. Although the analytical method itself has been proven to be robust, the data analysis for this unique method can be challenging and time consuming. It requires the extraction of several extracted ion chromatograms (EICs), manual integration, and many calculations that must be performed in Microsoft Excel.

In this application note, we demonstrate the use of the Agilent OAA for OpenLab CDS to analyze a LICA to determine its purity, assay, and impurity profile.

Experimental

Instrument parameters are adopted from the analytical method published by Rentel et al. at IONIS (Carlsbad, CA, U.S.) in 2022.[1] Table 1 highlights the relevant LC/MSD parameters. The sequence/injection work list is shown in Table 2. The method includes a four-level calibration for both UV and MS signals. Additionally, the workflow requires two different MS acquisition methods for each sample—standard and harsh. Note that specific MS acquisition may vary as they are dependent on the oligonucleotide sequence, and source settings should be optimized depending on empirical results.

The sample used for the analysis was a 16-mer ligand-conjugated antisense oligonucleotide that was supplied by AstraZeneca and prepared at approximately 0.1 mg/mL in water.

Data was acquired using Agilent OpenLab CDS software, version 2.8. Data processing was performed using Agilent OAA, version 1.0.

Table1. LC/MSD method parameters for Agilent OAA.

HPLC Parameters	
HPLC	Agilent 1290 Infinity II LC
Column	Hybrid C18, 3.5 µm 2.1 x 150 mm
Mobile Phase	A: 10% ACN, 5 mM tributylammonium acetate, 1 µm EDTA B: 80% ACN, 5 mM tributylammonium acetate, 1 µm EDTA
Flow Rate	0.25 mL/min
Column Temperature	50 °C
Gradient	45–80% B in 22 min
UV Detection	Variable wavelength detector, 260 nm
MS Parameters	
MSD	Agilent InfinityLab LC/MSD XT (product number G6135C)
Source	Electrospray ionization
Drying Gas Flow	13.0 L/min
Gas Temperature	255 °C (standard) 350 °C (harsh)
Nebulizer Pressure	25 psig
Capillary Voyage	4000 V
Mode	Negative
Scan	m/z 1347.3–1847.3
Scan Time	1149 ms (standard) 1200 ms (harsh)
Fragmentor	100
Gain Factor	2

Table 2. Sequence/injection work list.

Sample Name	Sample Type	Label	Injection Volume (µL)	Acquisition Method
Water Blank	Blank		25	Standard
Conditioning (Standard)	N/A		25	Standard
Conditioning (Harsh)	N/A		25	Harsh
Water Blank	Blank		25	Standard
Water Blank	Blank		25	Standard
Water Blank	Blank		25	Standard
Working Standard Solution (WSS), 10 µL	Calibration Standard		10	Standard
Working Standard Solution (WSS), 20 µL	Calibration Standard		20	Standard
Working Standard Solution (WSS), 25 µL	Calibration Standard	QC	25	Standard
Working Standard Solution (WSS), 30 µL	Calibration Standard		30	Standard
Working Standard Solution (WSS)	QC	QC	25	Standard
Sample (Standard	Sample	S1	25	Standard
Blank	Blank			
Sample (Harsh)	Sample	H1	25	Harsh
Blank	Blank		25	Standard
Working Standard Solution (WSS)	QC	QC	25	Standard
Wash	Sample		25	Standard

Results and discussion

OAA is a software add-on that processes OpenLab CDS data, acting as the front end to perform tasks necessary to complete the data analysis workflow. This includes manual steps like integration, signal overlays, and spectral examination, as well as calculations commonly performed within a spreadsheet program such as Excel. Figure 1 shows a high-level overview of each step of the process, from data acquisition to reporting and approvals.

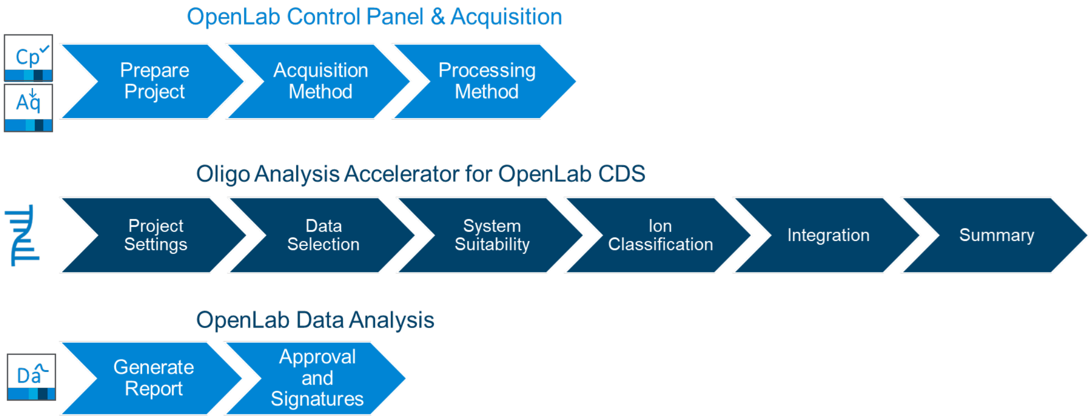


Figure 1. Purity, assay, and impurity profiling workflow by data system and steps within workflow. Acquisition occurs in Agilent OpenLab CDS software using the Agilent InfinityLab LC/MSD XT. Data processing is performed in the web application. Reporting is then performed back in OpenLab CDS.

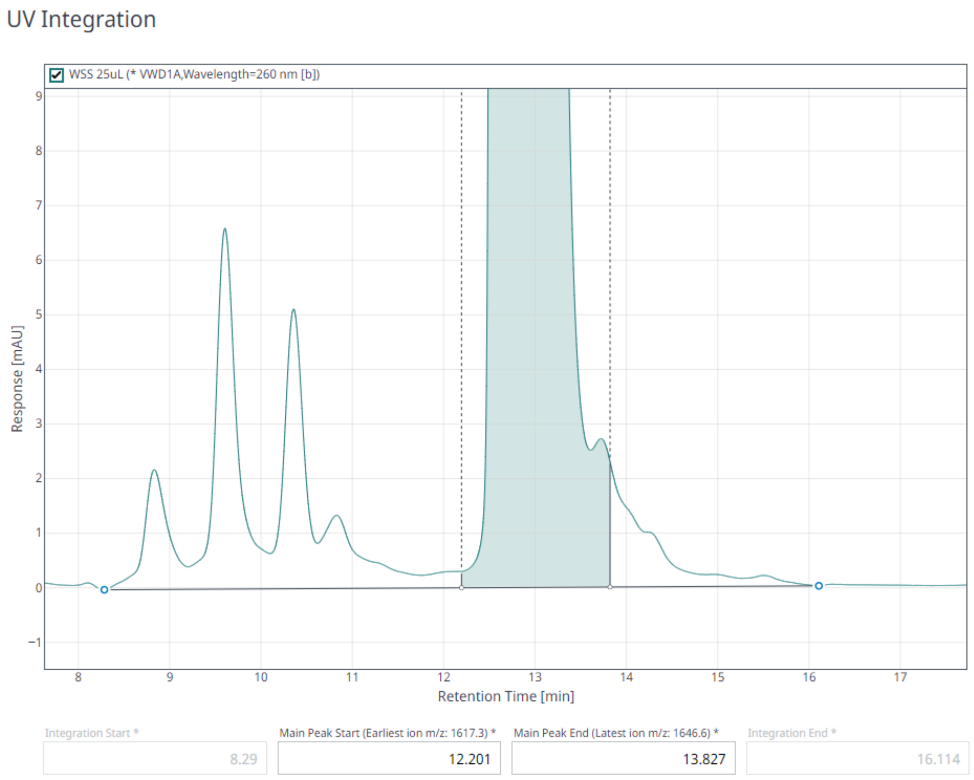
System suitability

After project settings and data selection has been configured, the first step in the data analysis workflow is determination of system suitability. A critical step in this analytical method is the proper integration of the UV main peak for QC standards. The integration and retention time of so-called early eluting impurities (EEI) and late eluting impurities (LEI) determine how the UV chromatogram is integrated. This is performed with EICs of predefined known EEIs and LEIs in the reference standard. Subsequently, a retention time offset is then considered due to the UV and MS signal delay in the flow path. This workflow is typically performed manually, with the chromatography data system (CDS) user extracting the known impurities from a list of ions, manually integrating each EIC, then determining which are the earliest and latest eluting impurities. Calculations are performed in a spreadsheet. This process is not only time consuming but can also be error prone due to manual transcription.

Conversely, the OAA algorithm automates EEI and LEI integration for the UV signal while automatically applying a retention time offset to the UV signal. Calculations are also performed within the application to determine system suitability so that the user does not need to transcribe them or generate a report to understand if the result set is valid.

Figure 1 shows the user interface (UI) for UV integration. Based on the OAA algorithm, it identified that the earliest eluting impurity is the ion at m/z 1617.3, which corresponds to the n-p(dMeC) impurity, or shortmer impurity missing methylated cytosine. The identified latest eluting impurity is the ion at m/z 1646.6, corresponding to the loss of a GalNAc + H2O. Given that the FLP is a LICA, the loss of the sugar moiety will lead to a more hydrophobic molecule, thus being later eluting by ion pair reversed-phase chromatography.

Should the user need to manually change the integration settings, they may do so directly in the web application UI. For example, commonly the n – 2 inflection point from the main peak is used instead of the earliest eluting impurity. In this case, the user can click and drag the dashed line in the interface and adjust as needed. The manual integration is noted in the audit trail for full traceability, with the audit trail indicating which user performed the manual integration; when the integration was performed; and how the integration was performed (that is, via the “DA API” or the web application).



Integration Start *: 8.29
Main Peak Start (Earliest ion m/z: 1617.3) *: 12.201
Main Peak End (Latest ion m/z: 1646.6) *: 13.827
Integration End *: 16.114

Figure 2. UV integration for system suitability in Agilent OAA. Impurities used for integration are displayed in the fields below the chromatogram. Main peak start integration is determined from the integration start for of m/z 1617.3, while subtracting the UV/MS offset. Main peak end integration is determined from the integration end for the m/z 1646.6, while subtracting the UV/MS offset.

Ion classification

The proceeding step after system suitability is the classification of mass peaks that exceed the threshold. Any peak with an m/z value less than the FLP and the height above 0.2%, or any peak with an m/z value more than the FLP and the height above 0.3%, must be classified based on the criteria described in Table 3.

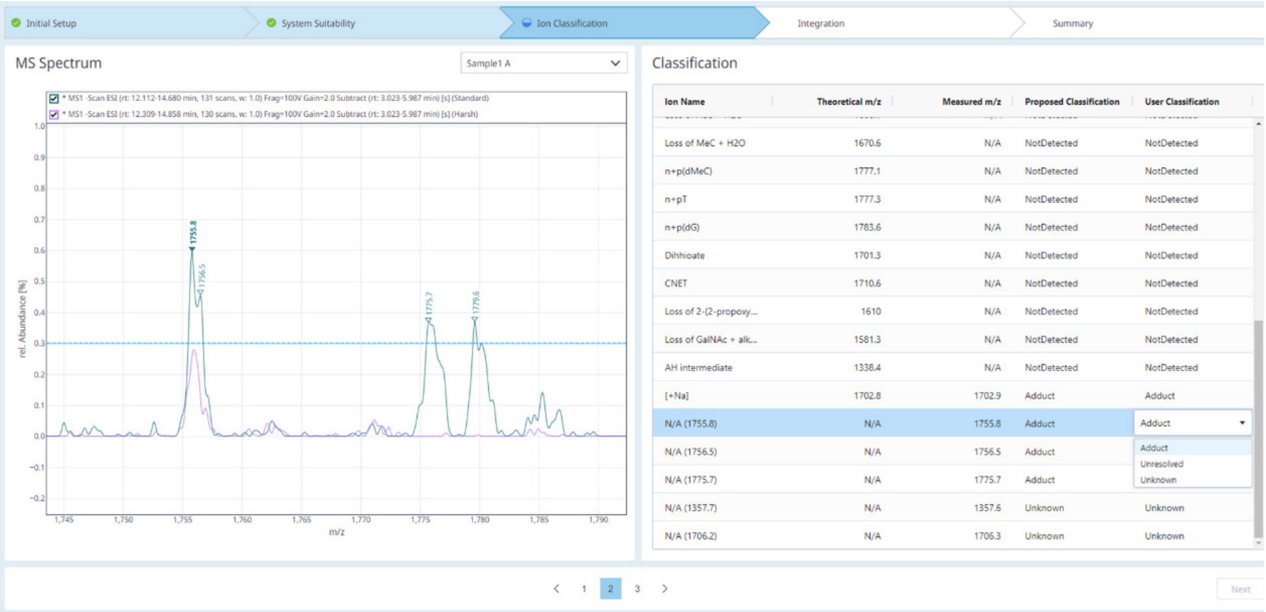
Table 3. Definitions for m/z values (ions) for ion classification step in Agilent OAA.

Ion Classification Name	Definition
Known Impurity	Known product/process-related impurity in the ion list; sufficient resolution between adducts (within m/z ± 0.5 of known ion)
Insufficiently Resolved	MS spectra peaks within m/z ± 1.0 resolution between known and unknown impurities
Adduct	Known adduct with no overlap (that is, m/z ≥ 0.5 resolution between unknown impurities)
Known Impurity / Possible Adduct	Known adduct with insufficient resolution between adduct and impurity (that is, m/z ≤ 0.5 resolution between adduct and known impurities); requires overlay of standard and harsh spectrum for confirmation
Unknown Impurity	Ion that exceeds threshold but does not match any of the values in the ion list; requires overlay of standard and harsh spectrum for confirmation
Not Detected	Known ion is below the threshold

Ion classification is a manual process because CDS platforms do not typically have built-in features for assessing spectra values above a threshold. As such, the user would need to manually identify m/z values above two different thresholds. Any values above the threshold would then be matched to the ion list, and each ion would be extracted appropriately. For accurate identification and classification of each known impurity / possible adduct and unknown impurity, an overlay of spectra from both injections of standard and harsh MS conditions is necessary, making visual inspection of ions that exceed the threshold increasingly difficult.

Conversely, OAA classifies these items automatically and reports theoretical m/z values against measured values. Additionally, the UI automatically overlays standard and harsh spectra from each sample, allowing for further inspection if needed. The user may then select a different classification from what the software algorithm has determined (Figure 3). This may be useful for regions in the full scan spectrum, where suspected impurities may have spectral overlap with other ions. For example, when a sample is suspected to contain postpeak n + 1, Na/K+ adducts (which may overlap), this can lead to misidentification even when comparing standard to harsh spectra values.

Once all ions are classified as either known or unknown, EICs are performed for each m/z value.



☑ * MS1 -Scan ESI (rt: 12.112-14.680 min, 131 scans, w: 1.0) Frag=100V Gain=2.0 Subtract (rt: 3.023-5.987 min) [s] (Standard)

☑ * MS1 -Scan ESI (rt: 12.309-14.858 min, 130 scans, w: 1.0) Frag=100V Gain=2.0 Subtract (rt: 3.023-5.987 min) [s] (Harsh)

Ion Name	Theoretical m/z	Measured m/z	Proposed Classification	User Classification
Loss of MeC + H2O	1670.6	N/A	NotDetected	NotDetected
n+p(dMeC)	1777.1	N/A	NotDetected	NotDetected
n+pT	1777.3	N/A	NotDetected	NotDetected
n+p(dG)	1783.6	N/A	NotDetected	NotDetected
Dihhioate	1701.3	N/A	NotDetected	NotDetected
CNET	1710.6	N/A	NotDetected	NotDetected
Loss of 2-(2-propoxy...	1610	N/A	NotDetected	NotDetected
Loss of GalNAc + alk...	1581.3	N/A	NotDetected	NotDetected
AH intermediate	1338.4	N/A	NotDetected	NotDetected
[+Na]	1702.8	1702.9	Adduct	Adduct
N/A (1755.8)	N/A	1755.8	Adduct	Adduct
N/A (1756.5)	N/A	1756.5	Adduct	
N/A (1775.7)	N/A	1775.7	Adduct	
N/A (1357.7)	N/A	1357.6	Unknown	Unknown
N/A (1706.2)	N/A	1706.3	Unknown	Unknown

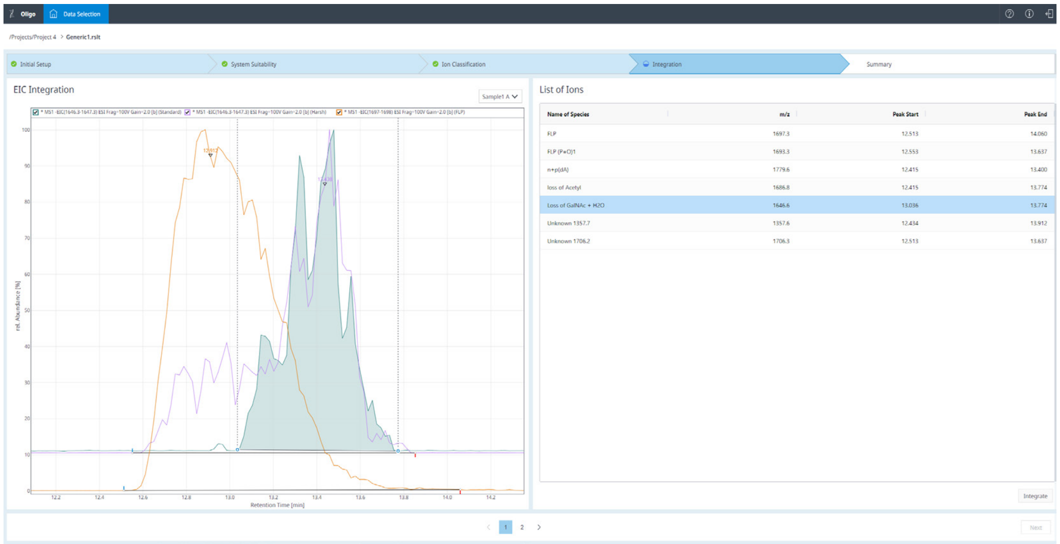
Figure 3. During the ion classification workflow, the user reviews software-proposed classification. Overlay spectra from both MS conditions are provided to facilitate confirmation of adducts. The user may also manually adjust the classification if needed.

Integration

The integration step evaluates each EIC to ensure proper quantitation. Resulting areas that are above the threshold of 0.2% relative quantitation are then used to determine the MS purity of the UV main peak, and thus the overall purity of the sample. Manual integration of EICs is required due to sample complexity and insufficient chromatographic resolution of closely related impurities.

To facilitate this workflow, OAA will generate three overlays in a graphical UI: FLP, EIC standard, and EIC harsh, as shown in Figure 4. The latest eluting impurity is again the more hydrophobic FLP with a loss of GalNAc and water, which results in nondistinct peaks. This may be either due to relative low amounts (≤ 1.0 % by relative peak area), or because of limitations in unit mass resolution leading to co-extracted impurities. In either case, the poor chromatographic resolution results in the need to manually integrate the peaks.

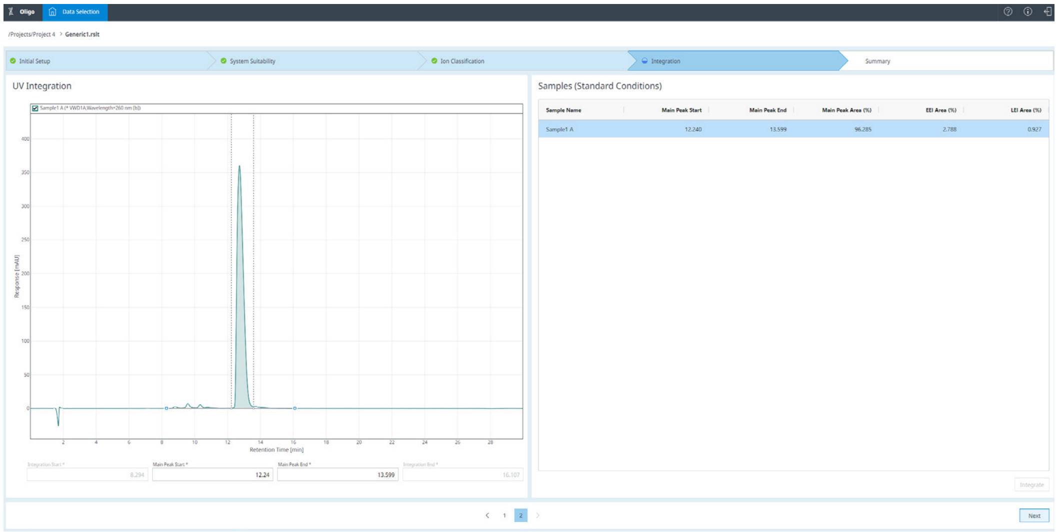
Once EIC peaks have been integrated for all samples, OAA will then apply the EEI and LEI settings for the UV main peak integration, similar to system suitability main peak integration. That is, EEI peak integration start is used for the start of the main peak integration, and LEI peak integration end is used for the end of main peak integration; UV and MS signal offsets occur automatically. Figure 5 shows that main peak integration is performed automatically based on EEI and LEI integration.



List of Ions

Name of Species	m/z	Peak Start	Peak End
FLP	1697.3	12.513	14.060
FLP (P=O)1	1693.3	12.553	13.637
n+p(dA)	1779.6	12.415	13.400
loss of Acetyl	1686.8	12.415	13.774
Loss of GalNAc + H2O	1646.6	13.036	13.774
Unknown 1357.7	1357.6	12.434	13.912
Unknown 1706.2	1706.3	12.513	13.637

Figure 4. Overlay of EICs for full-length product and standard/harsh conditions for "Loss of GalNAc + H20," which is an LEI. Overlays are automatically generated in the UI and the user can manually integrate peaks directly in the application.



Integration Start * 8.294
Main Peak Start * 12.24
Main Peak End * 13.599
Integration End * 16.107

Samples (Standard Conditions)

Sample Name	Main Peak Start	Main Peak End	Main Peak Area (%)	EEI Area (%)	LEI Area (%)
Sample1 A	12.240	13.599	96.285	2.788	0.927

Figure 5. After EIC integration is performed, sample UV integration is automatically generated based upon EEIs and LEIs. The user may also adjust the integration start and end points, if necessary.

Data summary and reporting

The final step in the workflow is to review initial purity and assay / label claim results (Figure 6). This includes the system suitability results shown in previous steps. It is important to note that the summary itself is not the report. An OpenLab CDS report template must still be used to report the results for review. Further, any electronic signatures and approvals occur within OpenLab Reporting, not in OAA. Again, this is because OAA leverages the already existing data compliance engine within the OpenLab CDS platform.

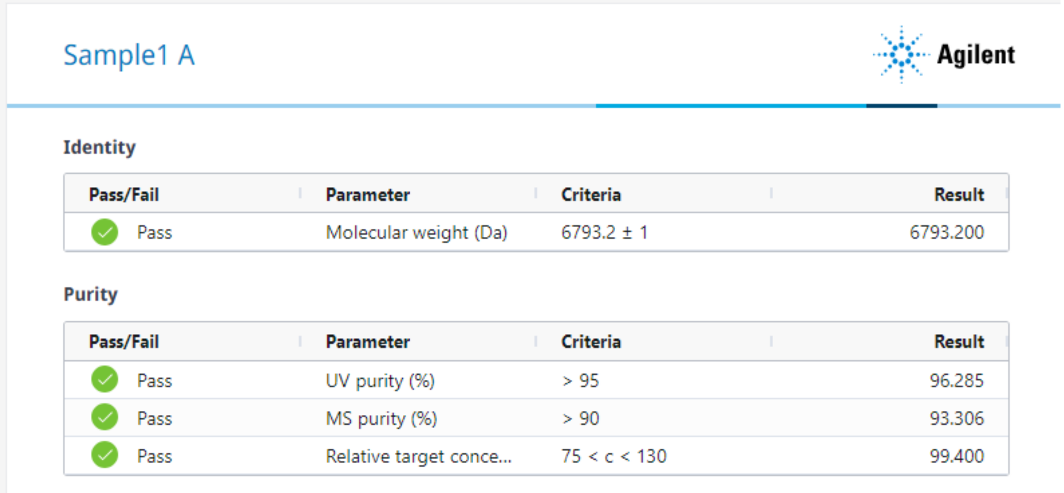
In addition, OpenLab Reporting has more extensive information on the sample, including relative quantitation of each of the impurities identified in the sample. Results for the tested oligonucleotide are specified in Table 4.

OpenLab Reporting allows for facile modification of the reporting template to customize the data that gets reported. This creates visibility to the reviewer to confirm data that is not shown in the web application workflow. An example would be the m/z values selected as EEI/LEI for integration of UV signal, along with their corresponding EICs.

In addition to purity, assay, and label claim calculations, the report can also provide calculations for ion categories such as n – 1 and abasic impurities. Additionally, an extensive impurity profile, with ions above either limit of detection or limit of quantitation, are also reported (Table 4).

Table 4. Impurity profile for sample.

Name	Measured Value	Area	Result (%)
FLP	1697.3	87402126.09	89.835
FLP (P=O)1	1693.1	501650.387	0.516
n+ p(dA)	1779.6	295681.246	0.248
Loss of Acetyl	1686.8	1343267.863	1.13
Loss of GalNAc + H2O	1646.4	560489.353	0.471
Unknown 1706.2	1706.2	378111.8885	0.317



Sample1 A

Identity

Pass/Fail	Parameter	Criteria	Result
✓ Pass	Molecular weight (Da)	6793.2 ± 1	6793.200

Purity

Pass/Fail	Parameter	Criteria	Result
✓ Pass	UV purity (%)	> 95	96.285
✓ Pass	MS purity (%)	> 90	93.306
✓ Pass	Relative target conce...	75 < c < 130	99.400

Figure 6. Summary page prior to report generation. Identity and purity results are reported, and system suitability results are available for review.

Conclusion

LC/MS analysis is the gold standard for determining the identity, purity, assay, and impurity profile of single-stranded oligonucleotide therapeutics such as ligand-conjugated antisense oligonucleotides (LICAs). The Agilent InfinityLab LC/MSD XT has the optimal selectivity and sensitivity for this analytical method. However, data analysis can be challenging due to the complex workflow, which traditionally requires multiple manual steps. In this application note, we demonstrate how the Agilent Oligo Analysis Accelerator for OpenLab CDS can be used to streamline data analysis and determine the purity, assay, and label claim for a LICA.

References

1. Rentel, C.; Gaus, H.; Bradley, K.; Luu, N.; Kolkey, K.; Mai, B.; Madsen, M.; Pearce, M.; Bock, B.; and Capaldi, D. Assay, purity, and impurity profile of phosphorothioate oligonucleotide therapeutics by ion pair–HPLC–MS. Nucleic Acid Ther. **2022**, 32(3), 206-220. https://doi.org/10.1089/nat.2021.0056

To learn more about Oligo Analysis Accelerator for OpenLab CDS, visit:

www.agilent.com/biopharma/oligo-analysis-accelerator

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