Application Note

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IP-RPLC Method Development for Oligonucleotide Impurities Analysis

Using ACD/AutoChrom integrated with Agilent OpenLab CDS on an Agilent 1290 Infinity III LC System with Agilent Altura Ultra Inert column hardware

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Abstract

The increasing complexity of therapeutic oligonucleotides demands robust, knowledge-driven analytical procedures capable of resolving closely related impurities while ensuring method robustness and lifecycle sustainability. Ion-pair reversed-phase liquid chromatography (IP-RPLC) remains the gold standard for oligonucleotide impurity analysis. However, method development is often challenged by adsorption of analytes to metal-containing surfaces, achieving sufficient selectivity to separate closely related oligonucleotide impurities such as n−1/n+1 species and modification variants from the full-length product (FLP), and ion-pair (IP) reagent selection and its impact on MS sensitivity, among other analytical challenges. This application note demonstrates the development of an IP-RPLC method for quantifying nusinersen impurities, by using the software ACD/Labs AutoChrom online integrated with Agilent OpenLab CDS. The Autochrom software was used to design, execute, and manage structured screening and optimization studies, while the chromatography data system (CDS) coordinated method execution and data acquisition. Critical method parameters, including different LC columns with low-adsorption LC hardware (Agilent Altura column line), and IP alkylamines, were systematically evaluated through screening and modeling-based optimization to identify optimum conditions that maximize resolution between the target impurities and the FLP. The results illustrate how software-enabled workflows, implemented within an AQbD-aligned development strategy, can accelerate method development and support informed selection of chromatographic conditions that deliver improved robustness, reproducibility, and impurity resolution for oligonucleotide analysis.

Introduction

Therapeutic oligonucleotides have become an established and rapidly expanding class of medicinal products, with applications ranging from rare genetic diseases to neurological and cardiovascular disorders.[1] Nusinersen, an antisense oligonucleotide approved for the treatment of spinal muscular atrophy (SMA), exemplifies both the clinical impact and the analytical challenges associated with this modality. During solid-phase synthesis and subsequent processing, a diverse range of impurities may arise, including truncated sequences, deletion and insertion variants, depurination products, backbone and base modifications, and closely related isobaric species 1. Due to their high polarity, multiple negative charges, and structural similarity among impurities, oligonucleotides present unique demands on chromatographic method development. IP-RPLC is widely used for oligonucleotide analysis and usually provides high resolving power for closely related sequence- and length-based impurities.[2,3] Retention is driven by a combination of hydrophobic and ion-exchange interactions, resulting in a dynamic complex mixed-mode separation mechanism. Positively charged alkylamines in the mobile phase condensate onto the negatively charged phosphate backbone of oligonucleotides, forming ion pairs that enhance interaction with the hydrophobic stationary phase while also modulating the extent of partitioning. In addition, IP reagents can adsorb onto the stationary phase, creating a charged surface layer that further contributes to retention and selectivity.[3] The balance between these interactions depends on several factors, such as the type and concentration of the IP reagent, column temperature, pH of mobile phase, and the stationary phase, directly impacting retention, selectivity, and peak shape. Electrostatic interactions can also promote non-specific adsorption to metal surfaces within the LC flow path, including column hardware and, to a lesser degree, system components.[4,5] This may result in reduced recovery, peak tailing, and poor reproducibility. Given that multiple analytical parameters influence performance, a systematic method development approach can support the identification of optimal conditions. In parallel, regulatory expectations for method development have been evolving toward a more systematic and risk-based framework. The principles outlined in ICH Q14[6] and the revised ICH Q2(R2)[7] emphasize the importance of understanding how method parameters influence analytical performance across the analytical procedure lifecycle, to aid in determining target conditions for the method and suitable control strategies.

Within this framework, structured screening and optimization studies are used to identify critical analytical method parameters (CAMPs), establish robust operating ranges, and support lifecycle management. The execution and interpretation of such studies can be facilitated by integrated software environments that coordinate experimental design, data acquisition, and data analysis while maintaining direct control through the CDS. ACD/Labs AutoChrom operates in direct communication with OpenLab CDS, enabling automated generation and execution of chromatographic methods, structured screening workflows, and centralized data handling without replacing scientific decision-making by the analyst. Within this configuration, AutoChrom functions as the experimental design and control layer, generating instrument methods and sample sequences that are executed by Agilent OpenLab CDS, allowing them to be saved within OpenLab CDS.

In this application note, a structured workflow is presented for the development of an IP-RPLC method for the quantification of nusinersen impurities, with emphasis on the practical integration of ACD/AutoChrom with OpenLab CDS. The study explores systematic screening of LC column chemistries, evaluation of low-adsorption LC hardware using the Altura column line, and comparison of commonly used IP alkylamines. This integrated approach demonstrates how coordinated software and CDS connectivity can support efficient experimentation, improve method understanding, and facilitate the selection of robust, fit-for-purpose chromatographic conditions for oligonucleotide impurity analysis.

Experimental

Materials

Nusinersen FLP and selected impurities n–1, n–2, n+1, and FLP with 16 phosphodiester linkages (16PO) and deaminated FLP (dFLP) were acquired from Eurogentec and are listed in Table 1. Acetonitrile (HPLC-S grade) and water (HPLC grade) were supplied by Biosolve. Alkylamines used for method development included diisopropylethylamine (DIPEA, 99.5% biotech grade, 496219), dibutylamine (DBA, ≥ 99.5%, 471232), and hexylamine (HA, 99%, 219703), which were acquired from Sigma-Aldrich. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, 99%, A1274722) was supplied by Thermo Fisher Scientific Chemicals.

Sample preparation

Two sample solutions were prepared at low and high concentration. The low concentration solution was prepared by spiking 0.5 mg/mL of FLP with 5% of each impurity in water. The high-concentration sample was prepared by mixing the FLP and impurities each at 0.2 mg/mL in water.

Instrumentation

Samples were run on an Agilent 1290 Infinity III LC System consisting of an Agilent 1290 Infinity III Flexible Pump (G7104A), an Agilent 1290 Infinity III Vialsampler (G7129B) with integrated sample thermostat, and an Agilent 1290 Infinity III Multicolumn Thermostat (G7116B). Detection was performed using an Agilent 1290 Infinity III DAD (G7117A) with an Agilent InfinityLab Max-Light Cartridge Cell, 10 mm (G4212-60008), followed by a single quadrupole Agilent InfinityLab LC/MSD iQ (G6160A). ACD/AutoChrom software served as the primary interface for method development, automatically converting experimental designs into OpenLab CDS-compatible instrument methods and sequence tables. Data acquisition was performed in OpenLab CDS version 2.7 (Agilent Technologies), under AutoChrom control, while data processing and evaluation were carried out within AutoChrom.

General method conditions

CAMPs assessed during the screening and optimization stages are summarized in Table 2 and Table 3. The final optimized method is described in Table 4.

Table 1. Sequence of nusinersen FLP and related impurities.

Sample	Sequence
Nursinersen FLP	5'<mU*mC*A*C*mU*mU*mU*mC*A*mU*A*A*mU*G*mC*mU*G*G>3'
Nusinersen Deaminated (dFLP)	< mU*mU*A*mU*mU*mU*mU*mU*A*mU*A*A*mU*G*mU*mU*G*G>
Nusinersen n+1	<mU*mC*A*C*mU*mU *mU*mC*A*mU*A*A*A*mU *G*mC*mU *G*G>
Nusinersen 16 PO	<mU mC A C mU mU mU mC A mU A A mU G mC mU G G>
Nusinersen n–1 (A)	<mU*mC*A*C*mU*mU*mU*mC*A*mU*A*mU*G*mC*mU *G*G>
Nusinersen n–2 (A/C)	<mU*A*C*mU*mU*mU*mC*A*mU*A*mU*G*mC*mU *G*G>

Legend: <>: 2'-O-MOE (2'-O-methoxyethyl); mU: 5-methyluridine; mC: 5-methylcytidine, *phosphorothioate linkage (if none: phosphodiester linkage).

Table 2. CAMPs assessed during the screening study.

CAMPs	Values
LC Columns[1]	– Agilent Altura HPH Oligo C18 with Ultra Inert technology, 2.1 × 100 mm, 2.7 µm (p/n 227215-702) – Agilent Altura ZORBAX Eclipse Plus with Ultra Inert technology, 2.1 × 150 mm, 1.8 µm (p/n 204125-308)
Flow Rate	0.4 mL/min
Mobile Phase A	15 mM DIPEA + 50 mM HFIP in water pH 8.6 15 mM DBA + 50 mM HFIP in water pH 8.6
Mobile Phase B[2]	15 mM DBA + 50 mM HFIP in acetonitrile 15 mM HA + 50 mM HFIP in acetonitrile
Gradient Slope[3]	DIPEA-DBA IP system: 10%–30% MPB in 20 min DIPEA-HA IP system: 10%–30% MPB in 20 min DBA-DBA IP system: 20%–50% MPB in 20 min DBA-HA IP system: 20%–50% MPB in 20 min
Injection	3 µL (high-concentration sample) 1.5 µL (low-concentration sample)
Needle Wash	Flush port, 3 s, water
Autosampler Temperature	5 °C
Column Temperature	60 °C
Detection: DAD	Full UV spectrum: 190–400 nm, 260 nm/4 nm Peak width > 0.1 min (2.5 Hz)
Detection: MS	ESI(–), gas temperature: 325 °C, gas flow: 11 L/min, nebulizer: 50 psi, capillary voltage: 3,500 V Data acquisition SIM mode: *m/z* 903 (n–2), *m/z* 959 (n–1), *m/z* 978 (16PO), *m/z* 1,017 (FLP and dFLP), *m/z* 1,074 (n+2)

[1] A 2-position/6-port valve was used for automated LC columns screening.

[2] If a higher concentration of HFIP is used, the use of acetonitrile may become critical, as it can lead to solubility issues.

[3] Two-minute equilibration and two blanks injected for conditioning the column before each experiment.

Table 3. Critical analytical method parameters (CAMP) assessed during the optimization study.

CAMPs	Values
LC Column	Agilent Altura HPH Oligo C18 with Ultra Inert technology, 2.1 × 100 mm, 2.7 µm (p/n 227215-702)
Flow Rate	0.4 mL/min
Mobile Phase A	15 mM DBA + 50 mM HFIP in water pH 8.6
Mobile Phase B	15 mM DBA + 50 mM HFIP in acetonitrile
Gradient Slope	20%–50% MPB in 20, 35, and 50 min
Injection	3 µL (high-concentration sample) 1.5 µL (low-concentration sample)
Needle Wash	Flush port, 3 s, water
Autosampler Temperature	5 °C
Column Temperature	60–65 °C
DAD	Refer to Table 1
MS	Refer to Table 1

Robustness assessment was also performed by selecting analytical factors and planning deliberated variation around their setpoints. Fractional factorial design was selected to study the following factors: flow rate (0.4 mL/min ± 0.04 mL/min), oven temperature (63 °C ± 2 °C) and initial gradient % (5% ± 1%). The four gradient time steps (min) and gradient % steps were also tested (± 10% of nominal value).

The final optimized IP-RPLC method is described in Table 4.

Table 4. Final IP-RPLC method.

Parameters	Values
LC Column	Agilent Altura HPH Oligo C18, 2.1 × 100 mm, 2.7 µm (p/n 227215-702)
Flow Rate	0.4 mL/min
Mobile Phase A	15 mM DBA + 50 mM HFIP in water pH 8.6
Mobile Phase B	15 mM DBA + 50 mM HFIP in acetonitrile
Injection	1.5 and 3 µL for low- and high-concentration samples, respectively
Needle Wash	Flush port, 3 s, water
Autosampler Temperature	5 °C
Column Temperature	63 °C
Detection DAD	260 nm/4 nm
Gradient Program	Time (min) MPA (%) MPB (%) 0 95 5 2 95 5 5 75 25 11 71 29 22 65 35 26 50 50 26.1 5 5 30 5 5

Results and discussion

The overall strategy implemented in ACD/AutoChrom consisted of LC column and mobile phase screening, followed by optimization of temperature and gradient, and then by robustness assessment of the optimized conditions. ACD AutoChrom was operated in online mode and directly integrated with OpenLab CDS, enabling automated creation of instrument methods, sample sequences, and execution of chromatographic runs without manual intervention. AutoChrom not only defined analytical runs based on the selection of relevant analytical parameters made by the user but also controlled the full sequence logic, including column equilibration, system conditioning, and injection order. This ensured that each experiment was executed under consistent and predefined system readiness conditions. OpenLab CDS served as the execution engine, carrying out all instrument commands generated by AutoChrom while maintaining full compliance with OpenLab CDS-controlled data acquisition workflows. This workflow enabled continuous knowledge management across the different stages of method development and is illustrated in Figure 1.

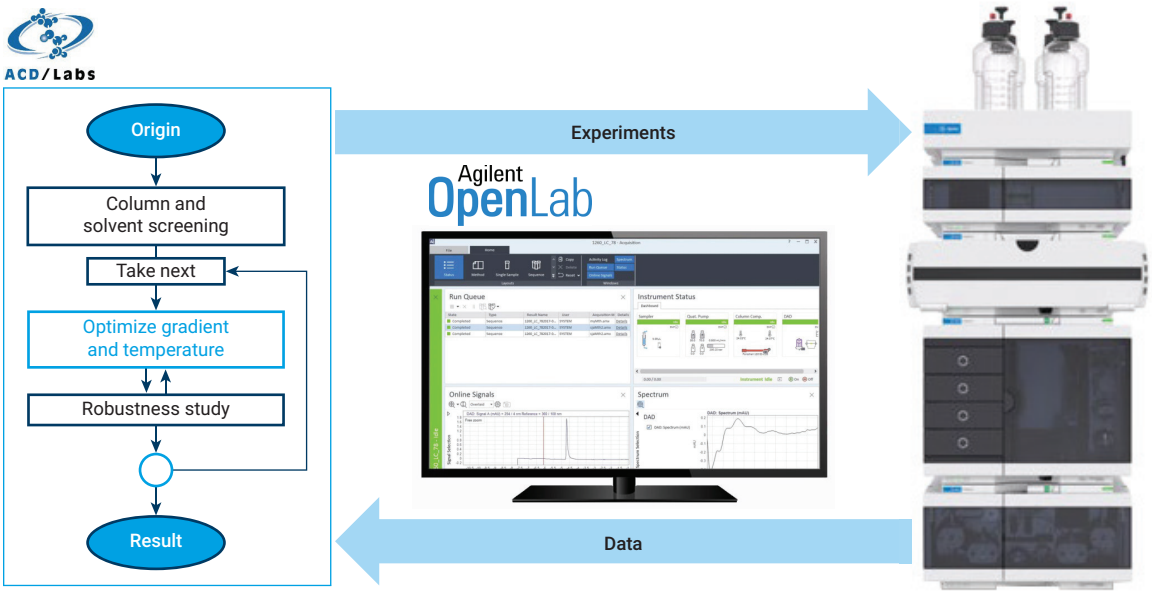


Figure 1. Example of method development strategy designed by ACD/AutoChrom and its integration with Agilent OpenLab CDS and an Agilent 1290 Infinity III LC System for data acquisition.

Given the strong dependence of oligonucleotide retention and selectivity on the nature of the IP system, the type of IP reagents was considered as a key driver of separation performance and selected as CAMP for the screening study. Alkylamines commonly classified as "weak" and "strong" IP reagents were selected. The screening considered two approaches: (i) constant IP concentration over time (e.g., mobile phase A (MPA) containing DBA/HFIP in water and mobile phase B (MPB) containing DBA/HFIP in acetonitrile, refer to as DBA-DBA), and (ii) weak-to-strong IP gradient[3] (e.g., MPA with DIPEA/HFIP in water and MPB with hexylamine (HA)/HFIP in acetonitrile, refer to DIPEA-HA).

Strong IP reagents (e.g., more hydrophobic alkylamines) are characterized by a higher affinity for adsorption onto the hydrophobic C18 stationary phase8, leading to the formation of a denser and more persistent charged interfacial layer. This promotes ion-exchange-like interactions between the oligonucleotide analytes and the IP reagents adsorbed on the stationary phase. In contrast, weak IP reagents adsorb to a lesser extent or a shorter time8, resulting in a thinner and more dynamic charged layer at the stationary phase surface. This shifts the balance between ion-exchange and hydrophobic interactions, enabling the exploration of alternative selectivity profiles. Consequently, these differences directly affect the dominant retention mechanism in IP-RPLC, influencing both analyte-stationary phase interactions and IP formation in the bulk phase. Based on these theoretical considerations, the selected IP systems were expected to differentially influence the separation of the active ingredient peak (FLP) from its structurally related impurities.

To efficiently explore this knowledge space, ACD/AutoChrom was used to build a structured screening strategy. The initial screening wave included two low-adsorption, C18-based columns (Altura Oligo HPH-C18 and Altura ZORBAX Eclipse Plus) and three IP reagents (DIPEA, DBA, and HA), with the objective of identifying conditions that maximize selectivity and resolution between the FLP and its known impurities. In total, eight experiments were planned by the software for the screening phase. For comprehensive peak characterization and confident impurity assignment, both full UV spectral data and MS data (selected ion monitoring mode (SIM)) were collected. UV spectra enabled peak-to-peak spectral comparison, while extracted ion chromatograms from the single-quadrupole MS confirmed impurity identities. This integrated UV-MS approach supported evaluation of selectivity across IP systems and informed method optimization. Following acquisition in OpenLab CDS, all data were automatically transferred to AutoChrom for processing and evaluation. AutoChrom performs peak detection, alignment, spectral peak matching, and impurity tracking across all experiments, enabling efficient comparison of screening results without manual data handling (Figure 2).

This analysis ensures alignment between experimental design, execution, and data interpretation within a single workflow.

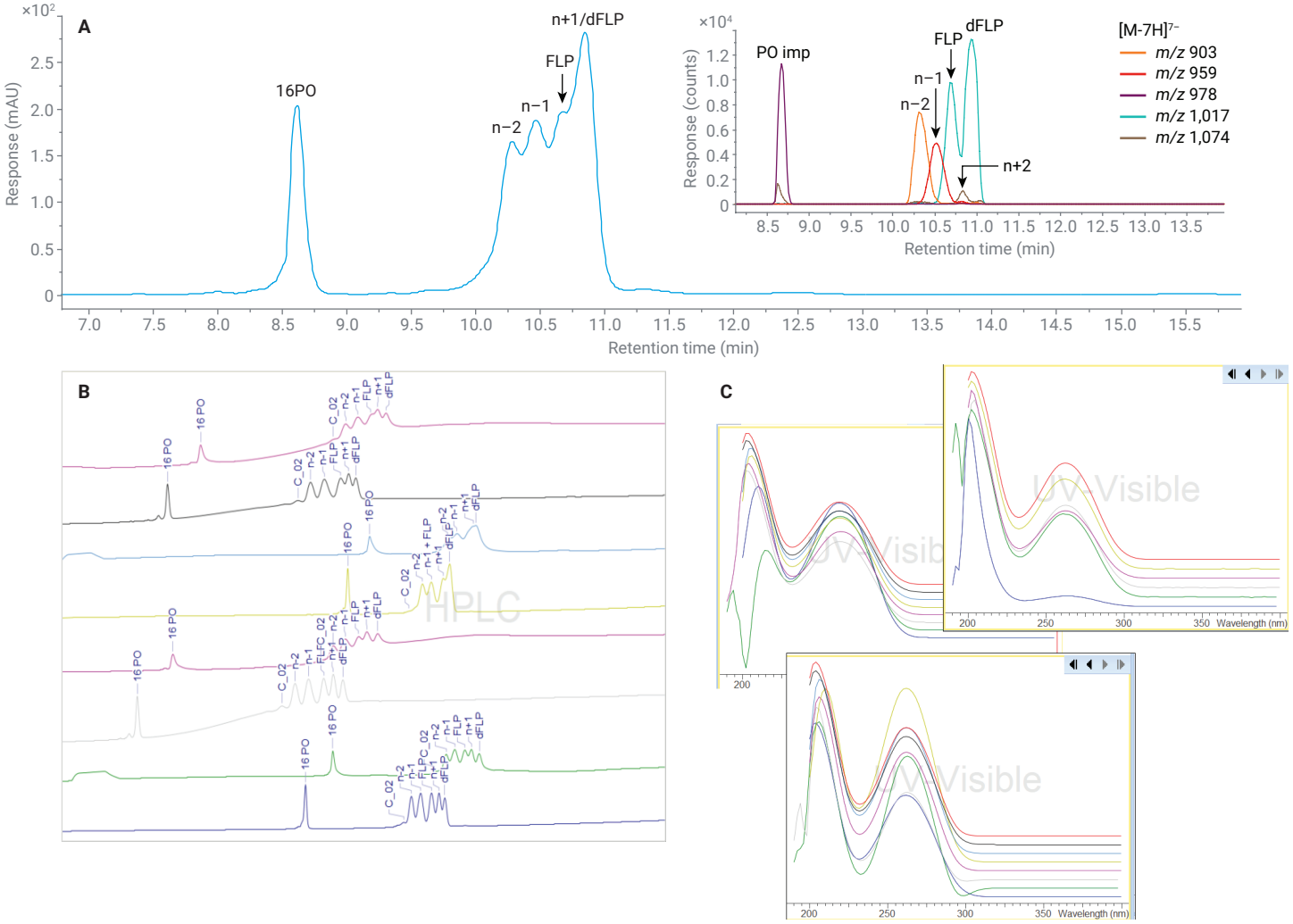


Figure 2. (A) UV chromatograms at 260 nm and the corresponding ESI(−) SIM traces obtained using the Agilent Altura ZORBAX Eclipse Plus system with MPA: 15 mM DBA and 50 mM HFIP in water (pH 8.6), and MPB: 15 mM HA and 50 mM HFIP in acetonitrile, as visualized in Agilent OpenLab CDS; (B) and (C) show all DAD chromatograms acquired during the screening stage together with the automatically matched UV spectra used for peak tracking, as visualized in ACD/AutoChrom and used for subsequent data processing.

Figure 3 shows the eight UV chromatographic profiles obtained during the screening experiments for the high-concentration sample. The results clearly demonstrate that IP systems containing DBA, used either in MPA or MPB, provide superior selectivity and higher resolution compared to the other conditions evaluated. All IP combinations employing HA in MPB resulted in coelution of the deaminated FLP impurity with the n+1 impurity. This observation supports previous hypotheses that chromatographic conditions favoring stronger hydrophobic interactions, relative to ion exchange contributions, may benefit the separation of species influenced by pronounced hydrophobic interactions, such as modifications variants (e.g., dFLP). HA, a linear alkylamine and the most hydrophobic IP reagent assessed in this study, likely enhanced ion-exchange-dominated interactions through strong adsorption to the C18 stationary phase. HA may have also masked hydrophobic interaction sites on the stationary phase, diminishing the contribution of differential hydrophobicity to the separation mechanism and leading to n+1 impurity and dFLP coelution. In addition to these observations, Altura Ultra Inert column hardware technology proved to be an effective strategy for mitigating non-specific adsorption between the analyte and metal traces, resulting in sharp and symmetrical peaks.

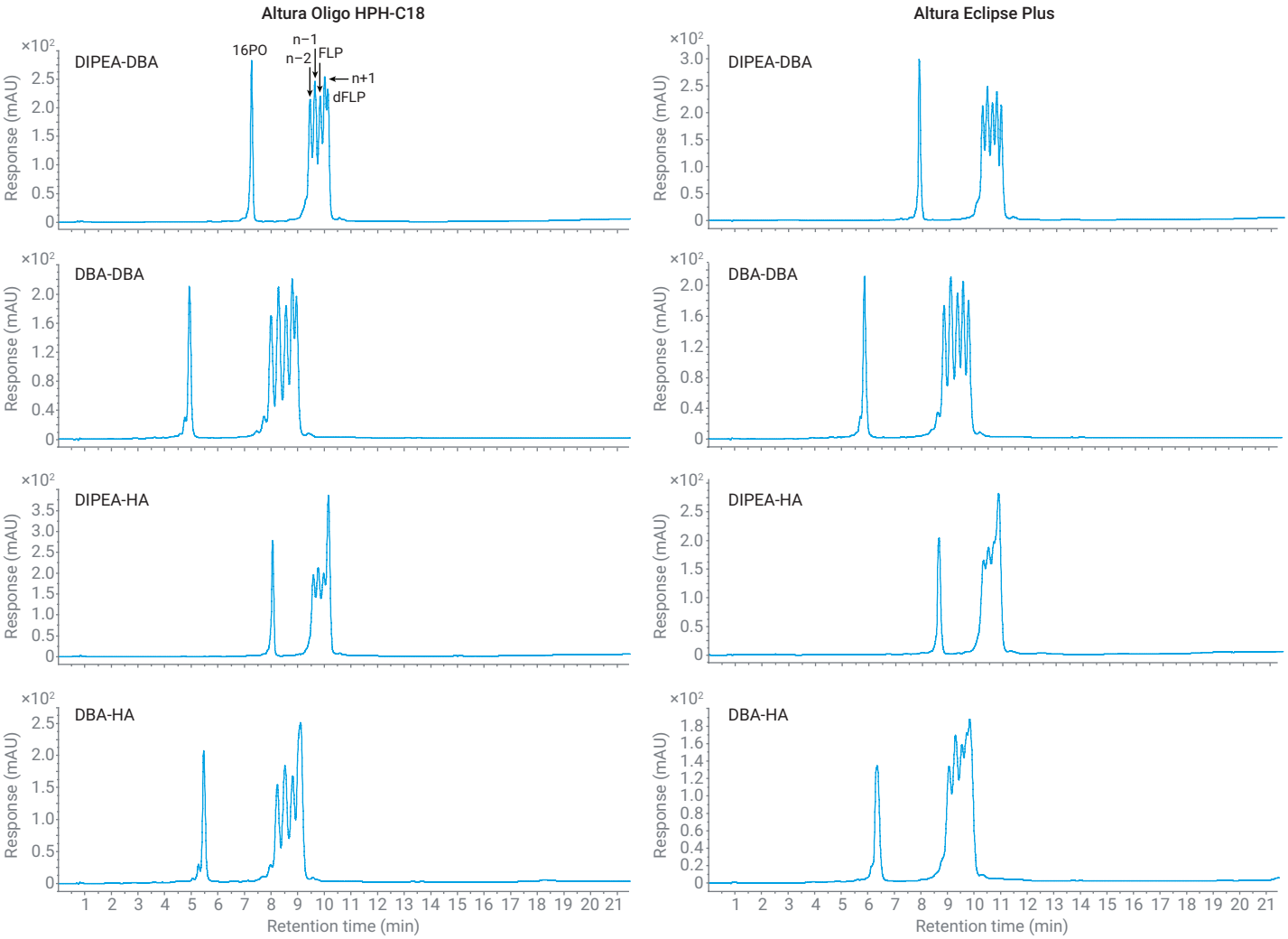


Figure 3. Acquired UV chromatograms of the high-concentration sample with different IP systems in MPA-MPB and LC columns during screening study.

The screening study indicated improved selectivity under the following conditions: Altura ZORBAX Eclipse Plus or Altura Oligo HPH-C18 columns using MPA and MPB containing 15 mM DBA (DBA-DBA). Under these conditions, the FLP peak and the five known impurity peaks were separated from one another and exhibited higher resolution (Rs) compared with the other IP systems evaluated. The Altura Oligo HPH-C18 column was selected for further optimization of the gradient program and column oven temperature, enabling the study of higher temperatures. Optimization was initiated by indicating the best-performing screening conditions into the AutoChrom software, which generated six additional experiments to evaluate the effects of oven temperature and gradient conditions. Upon completion of these optimization runs, retention time modeling was performed using the complete dataset, enabling in silico optimization of the gradient and temperature to maximize the resolution (Rs) between FLP and each impurity relative to its adjacent peaks. The optimization experiments generated by AutoChrom were directly deployed to OpenLab CDS without manual reconfiguration, ensuring continuity between the screening and optimization phases. Based on the prediction model, three additional experiments were performed to further refine the gradient model and confirm the predicted optimum separation conditions.

Within the LC Simulator interface, resolution maps and suitability limit graphs were used to visualize the impact of analytical conditions on critical method attributes (Figure 4). Predicted chromatograms were visualized across the resolution maps, aiding the design of conditions with optimal performance and enabling gradient modeling and establishment of a design space.

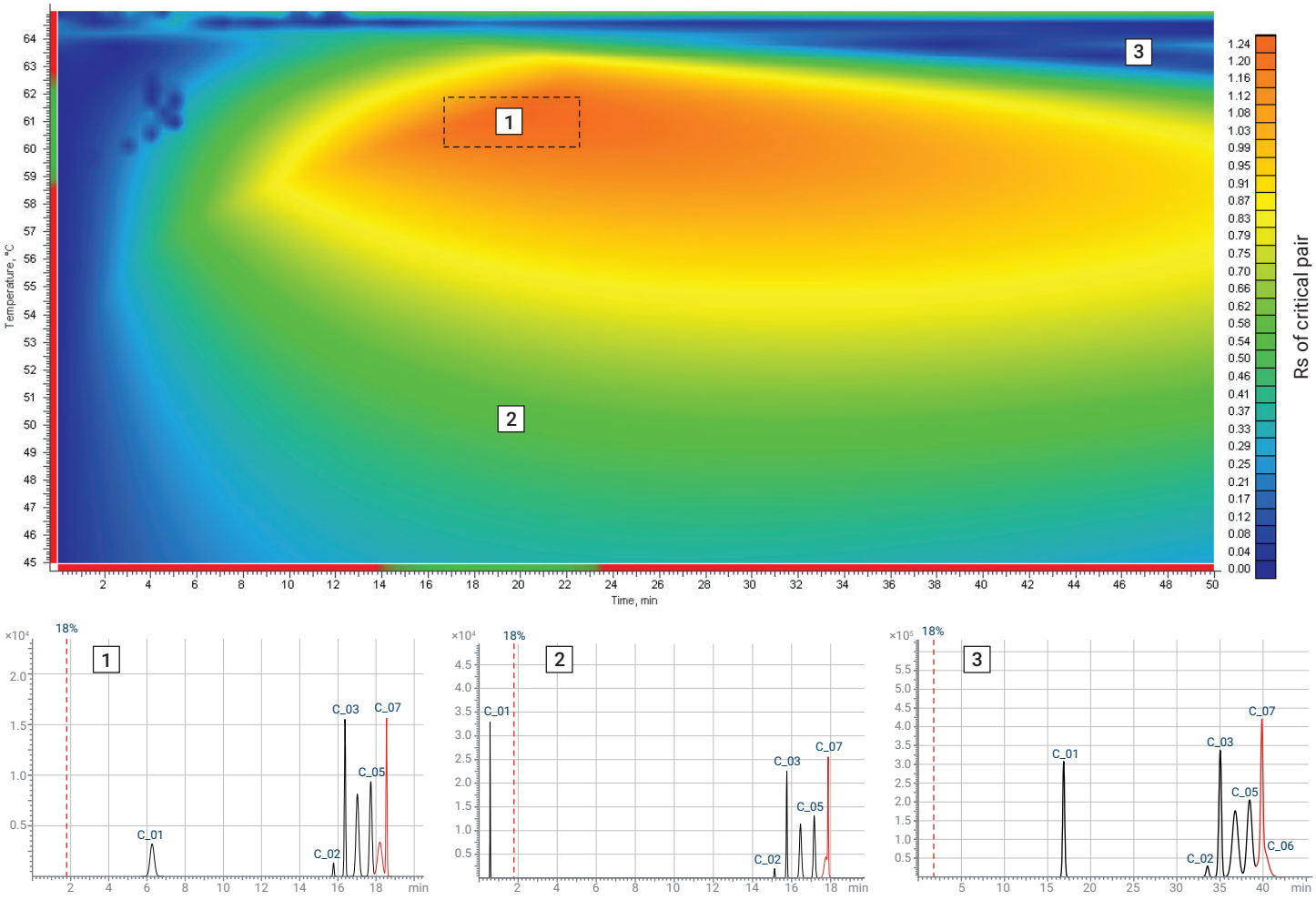


Figure 4. Predicted Resolution map representing a temperature range 59.0 to 65.0 °C and predicted chromatograms (1, 2, and 3) displayed in the ACD/LC simulator interface.

Based on the prediction model, a final method (Table 4) was designed to maximize Rs between target analytes. Figure 5 shows the chromatographic profiles obtained for low- and high-concentration samples analyzed using the final method.

The operational range defined during optimization served as the basis for the subsequent robustness assessment, where deliberate variations around the selected operating point were evaluated to confirm method stability within the established design space. Rather than relying solely on a one-factor-at-a-time approach, robustness was assessed using design of experiments (full factorial design) generated in AutoChrom, allowing the combined effects of temperature, flow rate, and gradient conditions to be systematically evaluated. The resulting chromatographic profiles (Figure 6) demonstrate that the method maintained consistent selectivity across the investigated conditions, with preservation of peak order and adequate separation between FLP and adjacent impurities. Despite intentional changes in the chromatographic parameters, no critical loss of Rs or peak merging was observed, confirming the robustness of the method within the defined operational range. Quantitative assessment of the final method conditions showed acceptable separation between the critical peak pairs, with Rs values of: Rs n−2/n−1 = 1.9, Rs n−1/FLP = 0.9, Rs FLP/n+1 = 1.6, and Rs n+1/dFLP = 1.0. These results support the reliability of the selected conditions and demonstrate that minor variations in method parameters are unlikely to impact analytical performance.

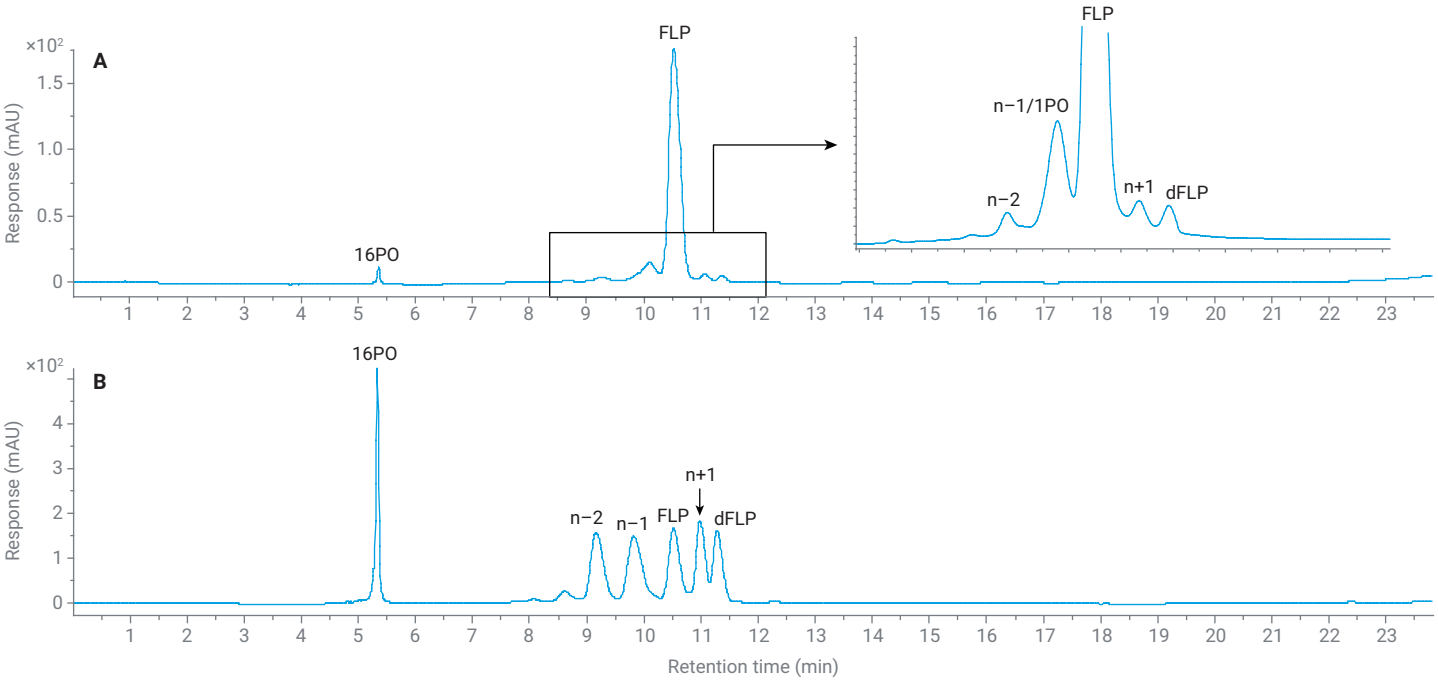


Figure 5. Chromatographic profiles of (A) low-concentration sample and (B) high-concentration sample acquired with the final method.

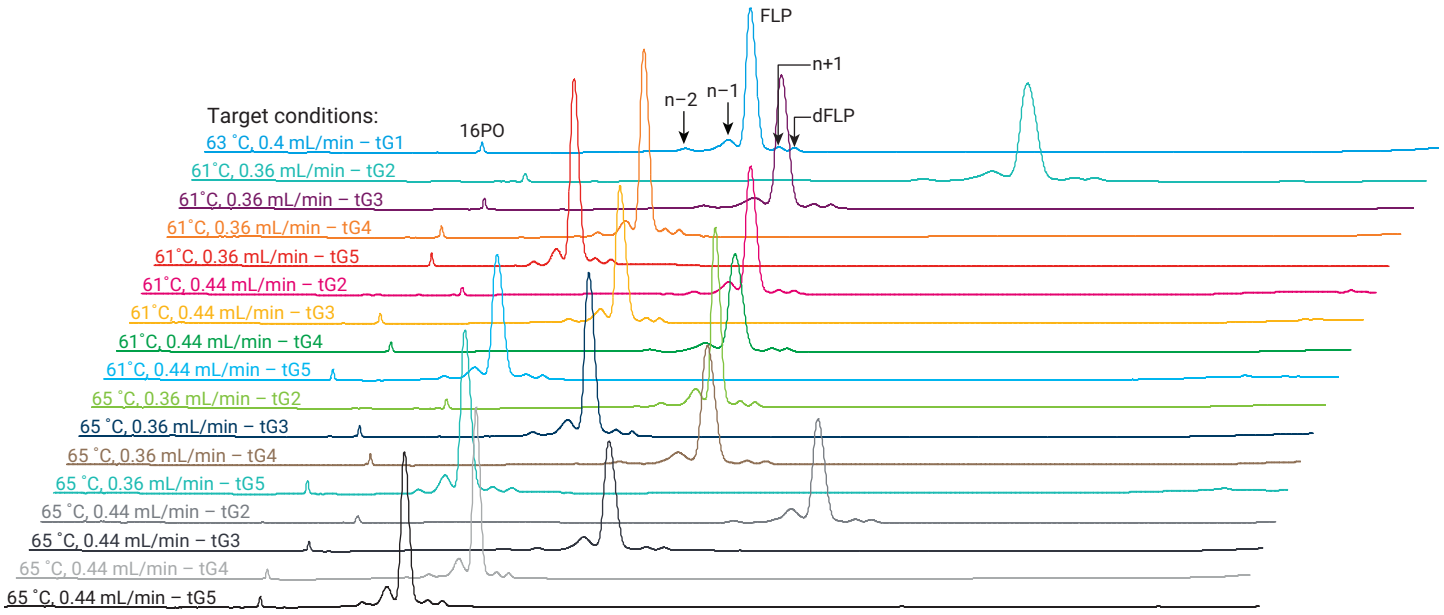


Figure 6. Chromatographic profiles obtained during robustness assessment using a DOE full factorial design (low-concentration mix solution).

Conclusion

This work demonstrates a structured analytical procedure development strategy combining systematic screening, model-informed optimization, and robustness assessment to support reliable impurity profiling of oligonucleotides. Integration of ACD/AutoChrom with Agilent OpenLab CDS enabled efficient experiment execution, automated data acquisition, and consistent data handling throughout development. Experimental designs generated in AutoChrom were translated into executable methods and sequences in OpenLab, including system preparation steps such as equilibration and conditioning, ensuring standardized implementation. All data were processed within AutoChrom, enabling direct comparison of conditions and data-driven decision-making. Knowledge generated during the workflow was captured alongside the experimental data, supporting traceability and documentation of screening outcomes, robustness evaluations, and final method conditions. This approach supports knowledge reuse, analytical procedure lifecycle management, and method refinement and transfer. The developed IP-RPLC method enabled robust separation of the nusinersen FLP from closely related oligonucleotide impurities, including n−1/n+1 species.

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