Application Note

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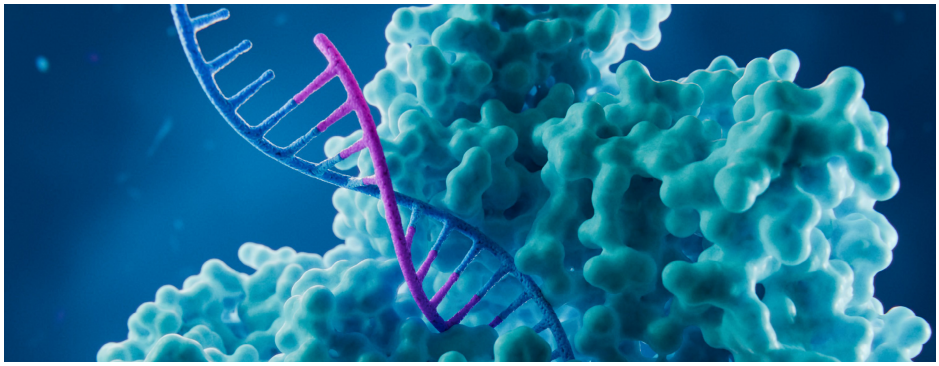
Complementary LC/MS and UV-Vis Spectrophotometry for siRNA Quality Control and Thermal Stability Assessment



Authors

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Abstract

Small interfering RNAs (siRNAs) are increasingly important therapeutic agents, which require robust analytical strategies to ensure product quality, duplex integrity, and thermal stability. In this study, ion-pair reversed-phase (IP-RP) liquid chromatography/mass spectrometry (LC/MS) and ultraviolet–visible (UV–Vis) spectroscopy thermal melt (T_m) analyses were integrated to provide comprehensive characterization of an siRNA duplex. LC/MS analysis was performed under both non-denaturing and denaturing conditions using the Agilent 1290 Infinity II Bio LC System coupled to the Agilent InfinityLab Pro iQ Plus mass detector. Under non-denaturing conditions, antisense (AS), sense (SS), and duplex species were baseline resolved and their molecular masses were confirmed with high accuracy. Thermal stability was evaluated using the Agilent Cary 3500 Multicell UV-Vis Spectrophotometer, yielding T_m temperatures of 82.5 °C in phosphate-buffered saline (PBS) and 46.8 °C in LC elution buffer, highlighting the strong influence of solvent composition on siRNA duplex stability. The results demonstrate that combining LC/MS structural analysis with UV-Vis thermal profiling provides a robust, orthogonal approach for siRNA quality control, suitable for development, characterization, and routine monitoring in biopharma applications.

Introduction

Small interfering RNAs (siRNAs) are short, double-stranded molecules that silence gene expression through RNA interference. Their ability to target specific genes has made siRNAs a promising class of therapeutics, driving growing interest and adoption for innovative treatment strategies in the pharmaceutical market.

Ion-pair reversed-phase chromatography (IP-RP) coupled with MS is the most common analytical strategy for siRNA characterization. By adjusting chromatographic conditions, siRNAs can be analyzed either as intact double-stranded duplexes under non-denaturing conditions, or as individual single strands under denaturing conditions. Combining both approaches provides a comprehensive structural assessment, ensuring accurate characterization of siRNA sequence integrity and duplex stability.

Thermal stability analysis by UV spectroscopy is commonly used to evaluate the conformational stability of nucleic acids. T_m is defined as the temperature at which 50% of the double-stranded molecule is unfolded (single stranded). The measurement serves as an indicator of siRNA duplex stability and is therefore a valuable parameter for their rational design and optimization.

In this application note, an siRNA sample was characterized under both denatured and non-denatured conditions using the Agilent InfinityLab Pro iQ Plus coupled with the 1290 Infinity II Bio LC System. Additionally, the T_m was determined using the 3500 Multicell UV-Vis Spectrophotometer. This study demonstrates how combining LC/MS and UV-Vis techniques (Figure 1) provides a complementary approach for siRNA quality control and structural assessment.

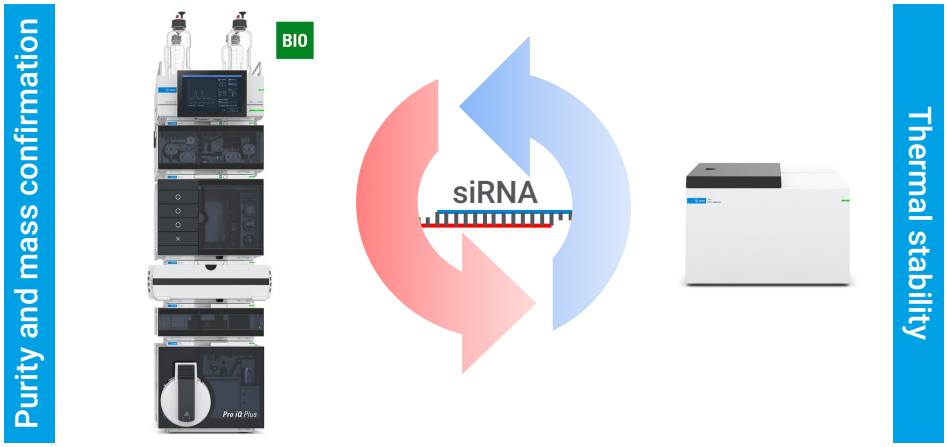


Figure 1. Integrated workflow for siRNA characterization that combines purity and mass confirmation with thermal stability analysis.

Experimental

Sample and sample preparation

Inclisiran single strands and duplex samples were purchased from MedChemExpress (NJ, USA). The samples were dissolved in RNase-free water at a concentration of 10 pmol/µL before injection into the LC/MS system.

The siRNA duplex concentration was approximately 20 µg/mL for UV-Vis measurement with either PBS (0.01 M phosphate, 0.0027 M KCl, 0.137 M NaCl, pH 7.4 at 25 °C) or HPLC elution buffer (15 mM TEA, 100 mM HFIP, 25% methanol). Neat PBS and elution buffer served as respective blanks.

Instrumentation

For LC/MS analysis, the 1290 Infinity II Bio LC coupled to the InfinityLab Pro iQ Plus mass detector system included:

- Agilent 1290 Infinity II Bio high-speed pump (G7132A)
- Agilent 1290 Infinity II Bio multisampler (G7137A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II variable wavelength detector (G7114B)
- InfinityLab Pro iQ Plus mass detector

T_m measurements were performed on an 3500 Multicell UV-Vis Spectrophotometer using Agilent quartz semimicro cuvettes (part number 50636559) with an Agilent Temperature Probe (part number G9889-60005), 800 µL volume, and 10 mm pathlength. Drops of mineral oil were layered on the sample using an Agilent mineral oil dropper (part number FSSMO15) to minimize evaporation.

Software

Agilent OpenLab CDS (version 2.8) and Agilent Cary UV Workstation software (version 1.6) were used for data acquisition and analysis.

LC/MS analysis

Tables 1 and 2 list the acquisition parameters for the LC and MS instruments. Table 3 lists the deconvolution settings that were specified in Agilent OpenLab CDS Data Analysis software

UV-Vis T_m measurements

The UV-Vis instrument parameters are listed in Table 4. All measurements were conducted in triplicate. An incuvette temperature probe was inserted into each sample cuvette to provide feedback to enable control of the experimental temperature.

Table 1. LC parameters.

Parameter	Agilent 1290 Infinity II Bio LC System
Column	Altura Oligo HPH-C18, 2.1 × 50 mm, 2.7 µm (p/n 227205-702)
Thermostat	8 °C
Solvent A	15 mM TEA and 100 mM HFIP in water
Solvent B	Solvent A:methanol 50:50
Flow Rate	0.5 mL/min
Gradient	Time (min) %B 0.0 10 10.0 70
Post Time	4 min
Injection Volume	2 µL, with 10-second needle flush port wash of 50% methanol
Column Temperature	30, 40, 50, and 60 °C

Table 2. MS data acquisition parameters.

Parameter	Agilent InfinityLab Pro iQ Plus LC/MS
Source	Agilent JetStream ESI Source
Polarity	Negative
Gas Temperature	275 °C
Gas Flow	12 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	2,000 V
Fragmentor	120 V
Scan Mode	Scan only
Scan Range	*m/z* 700 to 3,000 in profile mode
Scan Time	0.54 Hz

Table 3. LC/MS spectral deconvolution parameters.

Parameter	Value
Spectrum Extraction Mode	Peak apex spectrum
Background Mode	Spectrum at peak start and end
Use *m/z* Range	Disabled
Low Molecular Weight	7,000
High Molecular Weight	18,000
Maximum Charge	33
Minimum Peaks in Set	6
MW Agreement (0.01%)	5
Absolute Noise Threshold	1,000
Relative Abundance Threshold	15%
MW Algorithm	Curve Fit
MW Algorithm Threshold	40
Envelope Threshold	50

Table 4. UV-Vis spectrophotometer parameters.

Parameter	Agilent Cary 3500 Multicell UV-Vis Spectrophotometer
Wavelength	260 nm
Signal Averaging Time	1 s
Data Interval	0.5 °C
Start Temperature	25 °C
End Temperature	90 °C
Return Temperature	25 °C
Temperature Ramp Rate	5 °C/min
Number of Stages	1
Temperature Control	Temperature probe
Smoothing Filter	11
Smoothing Data Interval	0.5 °C
Derivative Filter	11
Derivative Data Interval	0.5 °C

Results and discussion

siRNA stability at various temperatures

Quality control of siRNA duplexes is often performed using an IP-RP LC method. Unlike antisense oligonucleotide (ASO), the siRNA duplex structure is sensitive to temperature and mobile phase composition. Denaturation of the duplex can occur on-column under high temperature conditions.[1] To find the optimal non-denaturing temperature for our siRNA, a series of column temperatures from 30 to 60 °C in 10 °C intervals was tested. The results in Figure 2 indicate that the siRNA remained in duplex from at column temperatures of 30 and 40 °C. With increasing column temperature, the siRNA eluted earlier despite use of an identical gradient. This behavior reflects the enhanced mass-transfer efficiency and reduced viscosity of the mobile phase at elevated temperatures, which together decrease analyte retention and accelerate chromatographic elution. The broadened peak of siRNA under non-denatured conditions can be attributed to the partial separation of its different phosphoramidate diastereomers.[1]

The onset of duplex melting happened at column temperatures between 40 and 50 °C. At a column temperature of 60 °C, siRNA is almost fully denatured to single strands as depicted in the overlaid chromatogram of antisense strand (AS), sense strands (SS) and duplex (Figure 3). Meanwhile, the wide siRNA peak measured under denatured conditions reflects its various denatured conformations and the diastereomer content.

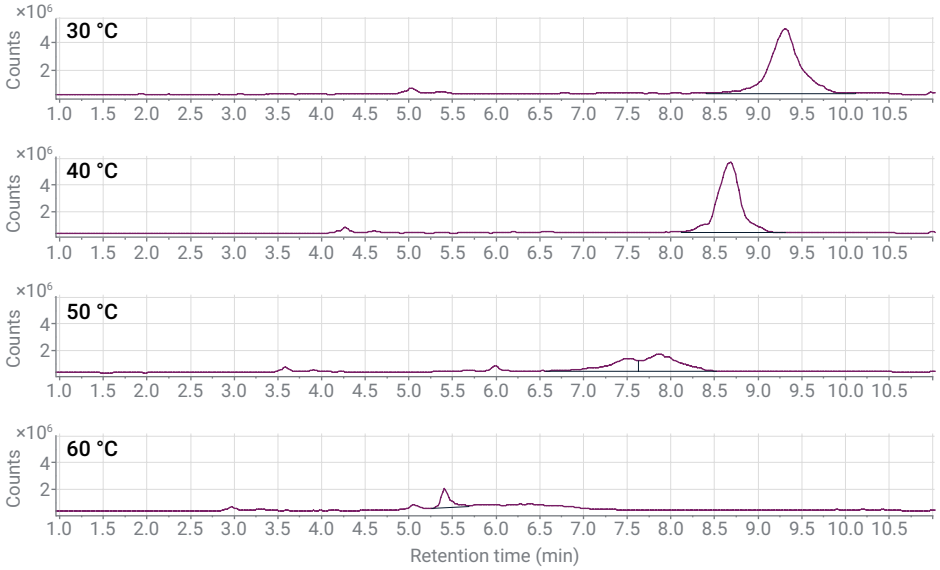


Figure 2. Chromatograms of the IP-RP LC separations of siRNA at column temperatures of 30, 40, 50, and 60 °C (top to bottom).

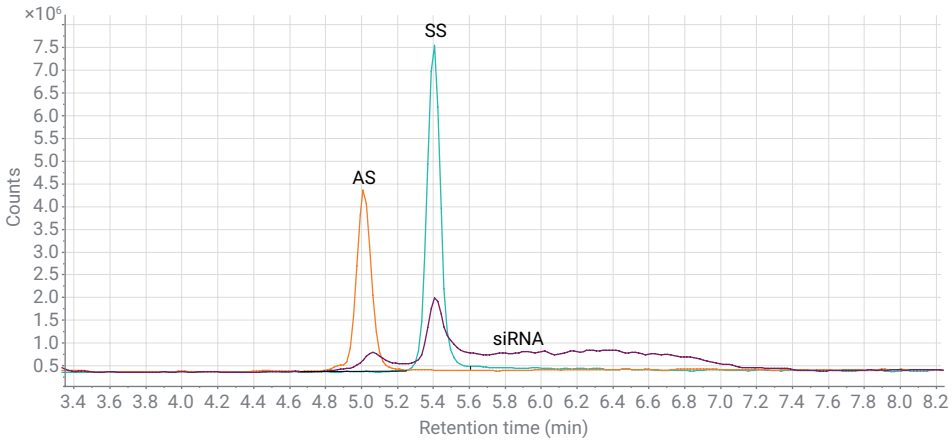


Figure 3. Overlaid IP-RP chromatograms of antisense (AS), sense strands (SS), and siRNA at a column temperature of 60 °C.

Separation and identification of AS, SS and siRNA at 30 °C

The goal of siRNA duplex analysis is to identify the presence of single-stranded siRNA components in the siRNA. Under non-denatured conditions and a 30 °C column temperature, the AS and SS were well separated from siRNA duplex (Figure 4). The AS eluted earlier than the SS because the SS is conjugated to the tri-antennary GalNAc ligand, which increases its hydrophobicity under reversed-phase conditions and therefore prolongs its retention time.

LC/MS analysis was performed to determine the molecular weight of the individual single strands and the duplex, thereby confirming the identities of all components. The MS spectra and measured masses are shown in Figure 5 and Table 5, respectively.

AS and SS display a similar charge state distribution pattern (Figures 5A and 5B). After deconvolution, the measured molecular weight (MW) of each single strand matched their respective theoretical MW with of delta mass of less than 2 Da. No regulatory or vendor guideline specifies an absolute Da-based acceptance criterion for oligonucleotide mass confirmation. Industry practice establishes laboratory-specific tolerances, typically on the order of 1–5 Da for 7–8 kDa oligos, depending on the MS platform and deconvolution method used.

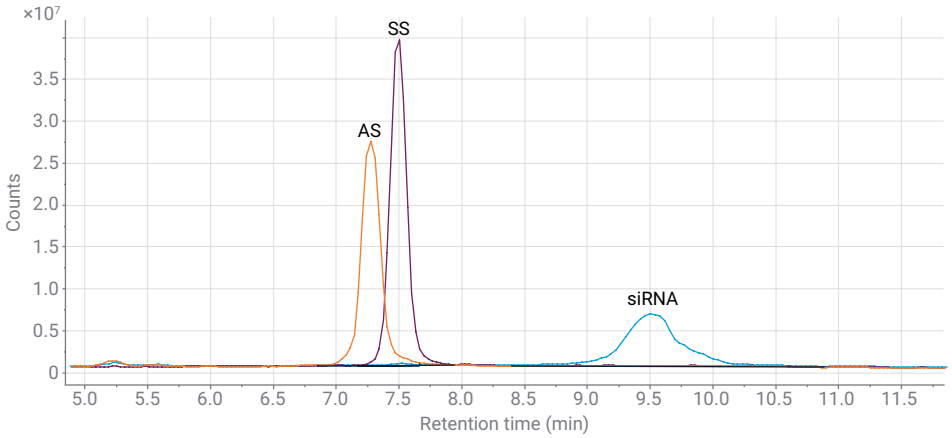
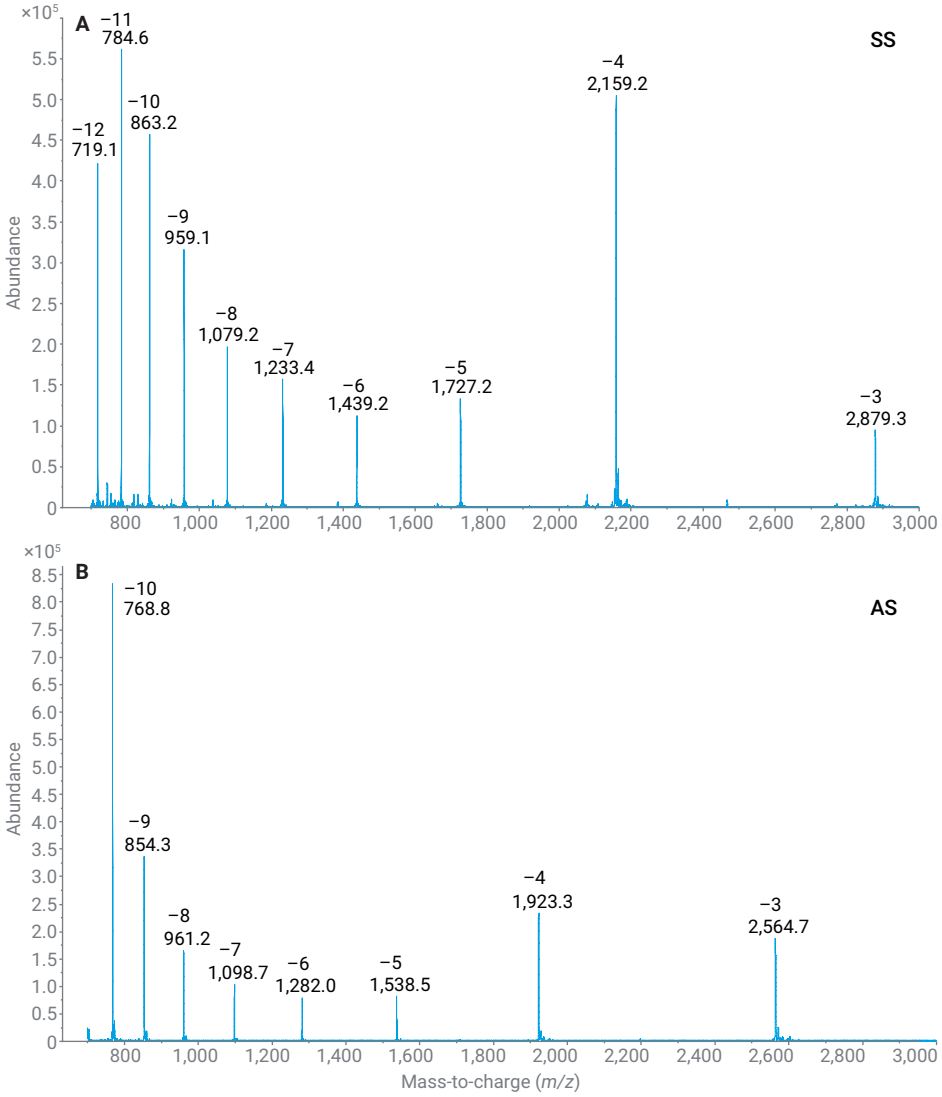


Figure 4. Overlaid IP-RP LC chromatogram of antisense (AS), sense strands (SS),and siRNA at a column temperature of 30 °C.



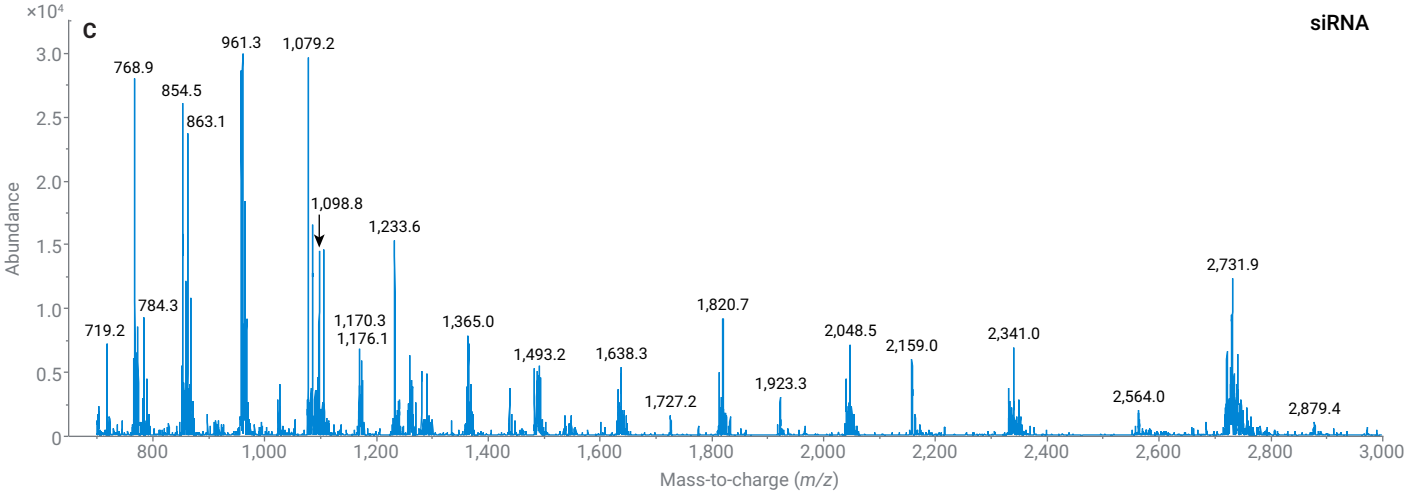


Figure 5. Mass spectra of SS (A), AS (B), and siRNA (C). Charge states were labeled corresponding to ions in the SS and AS spectra.

Table 5. MW of AS, SS, and the siRNA sample.

	Theoretical MW (Da)	Observed MW (Da)
SS	8,642.5	8,641.14
AS	7,699.6	7,697.79
siRNA	16,342.1	8,640.93 7,698.43 16,397.10 8,697.75 7,755.04

siRNA duplexes are known to disassociate into single strands under heated and high-energy electrospray ionization (ESI) conditions.[2] As a result, the mass spectrum of the siRNA sample (Figure 5C) represents a mixture of signals originating from two single strands and the intact duplex. Since the charge states of these species are very similar—and in some cases even overlap—the resulting spectrum appears highly congested. In addition, adduct ions are present in the spectrum, contributing to the overall complexity of the signal. Despite these challenges, all components were successfully identified using Agilent MS Spectral Deconvolution for OpenLab CDS Deconvolution software. The software's advanced algorithms effectively resolved overlapping charge states and adduct-rich signals, demonstrating its strong capabilities for interpreting highly complex oligonucleotide spectra.

3500 Multicell UV-Vis Spectrophotometer T_m measurement

Thermal stability is a critical parameter for siRNA activity.[3] The UV-Vis technique is used to monitor changes in UV absorbance as a function of temperature, which reflects structural unfolding or denaturation. T_m values are strongly affected by duplex concentration, duplex sequence, oligonucleotide length, chemical modifications, and buffer compositions.[1] The T_m of the nucleic acids in this study were measured at an siRNA duplex concentration of 20 µg/mL under both the conditions tested.

UV-based T_m analysis is simple, rapid, and widely applied for stability comparison under different formulation conditions. Melting temperatures measured in LC mobile phases containing organic solvents can differ significantly from those obtained in physiologically relevant aqueous formulation buffers. In this study, the T_m of inclisiran was compared in an aqueous buffer (PBS) and in LC elution buffer conditions. Figure 6 shows the melting curves obtained in both buffers. The T_m was determined to be 82.5 °C in PBS and 46.8 °C in organic elution buffer, demonstrating the pronounced effect of organic solvents on siRNA thermal stability (T_m values were determined as first derivative). UV-Vis-derived T_m values for both solvents are summarized in Table 6. These results were well corroborated by LC-based studies, which showed good agreement with the UV-Vis method.

The 3500 UV-Vis Multicell Spectrophotometer offers 10-year lamp warranty for long-term reliability, and high throughput due to its multicell design. The instrument is ideal for temperature-controlled experiments, particularly when configured with the precise temperature-sensing probe. Compliance software ensures secure data management and facilitates regulatory compliance.[4]

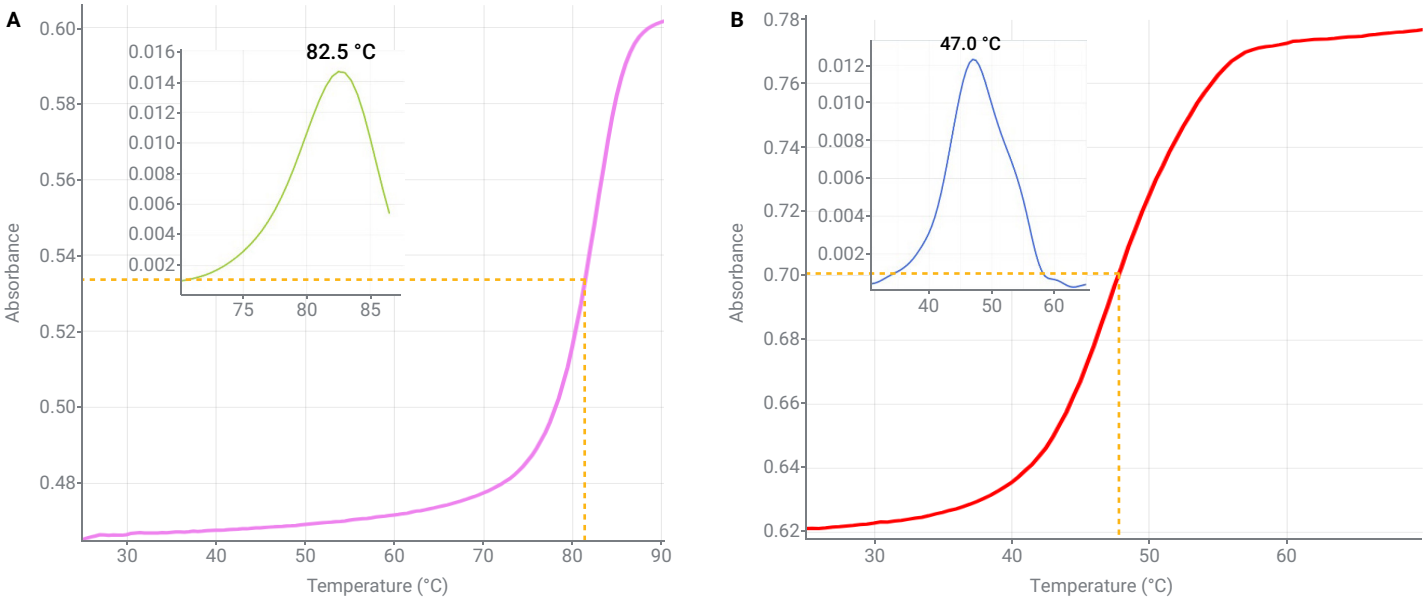


Figure 6. Inclisiran absorbance versus temperature and the corresponding first derivative (inset) as a function of temperature at two conditions: (A) PBS and (B) organic elution buffer.

Table 6. Measured T_m values for inclisiran at different buffer conditions.

Buffer Description	T_m 1 (°C)	T_m 2 (°C)	T_m 3 (°C)	Average T_m (°C)	Standard Deviation T_m (°C)
Phosphate Buffer Saline (PBS)	82.5	82.5	82.5	82.5	0
15 mM TEA and 100 mM HFIP in Water+ 25% Methanol	47	47	46.5	46.8	0.20

Conclusion

This application note demonstrates that integrating LC–MS and UV–Vis T_m analysis provides a powerful, complementary strategy for siRNA structural characterization and quality control. By evaluating the effects of column temperature on chromatographic results, we identified 30 to 40 °C as optimal non-denaturing conditions for maintaining duplex integrity in IP-RP LC separations, while enabling effective resolution of AS, SS, and duplex species. Mass confirmation showed excellent agreement between measured and theoretical molecular weights, supporting reliable identification of individual strands and duplex components. UV–Vis analysis further revealed substantial differences in T_m between physiological buffer (PBS) and LC elution solvent, underscoring the critical role of formulation conditions in siRNA stability. Overall, the combined workflow offers a robust analytical platform for siRNA development, providing orthogonal confirmation of structural integrity, accurate mass determination, and quantitative thermal stability assessment. This integrated approach is well-suited for method development, comparability studies, and routine QC of therapeutic siRNA products.

References

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2. Huang, T. Y.; Liu, J.; Liang, X. R.; Hodges, B. D. M.; McLuckey, S. A. Collision-Induced Dissociation of Intact Duplex and Single-Stranded siRNA Anions. *Anal. Chem*. **2008**, *80*, *22*, 8501–8508
3. Berezhnoy, A.; Brenneman, R.; Bajgelman, M.; Seales, D.; Gilboa, E. Thermal stability of siRNA modulates aptamerconjugatedconjugated siRNA inhibition. *Mol. Ther. Nucleic Acids* **2012**, *1*, e51.
4. Agilent Technologies. Interview: Evaluating an Innovative Analytical ID Testing Strategy for Oligonucleotides. *Agilent Technologies case study*, publication number 5994-5144EN, **2022**.

Further information

- Cary 3500 Multicell UV-Vis Spectrophotometer
- Cary UV Workstation software
- UV-Vis Spectroscopy & Spectrophotometry FAQ

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