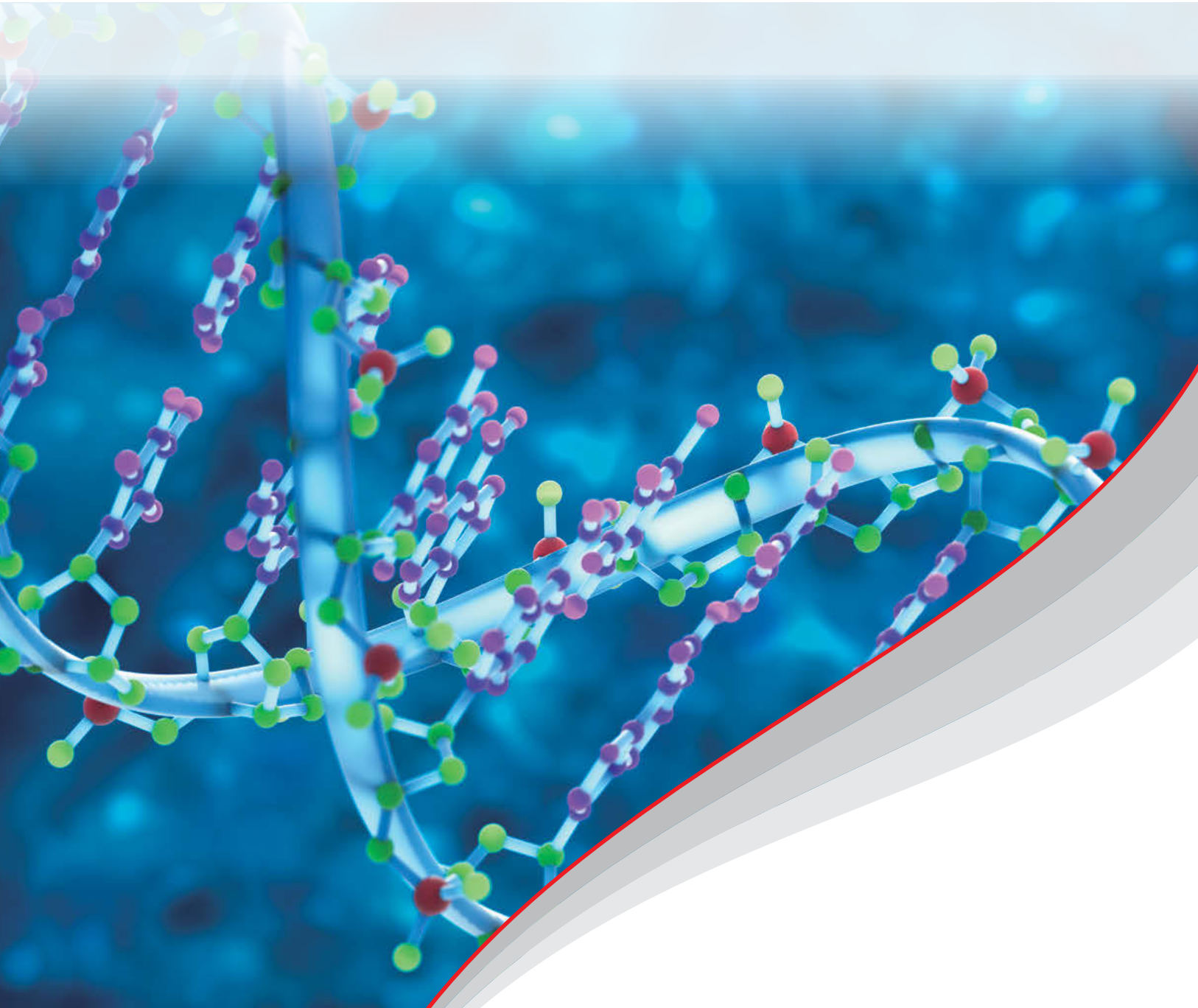
C10G-E097B

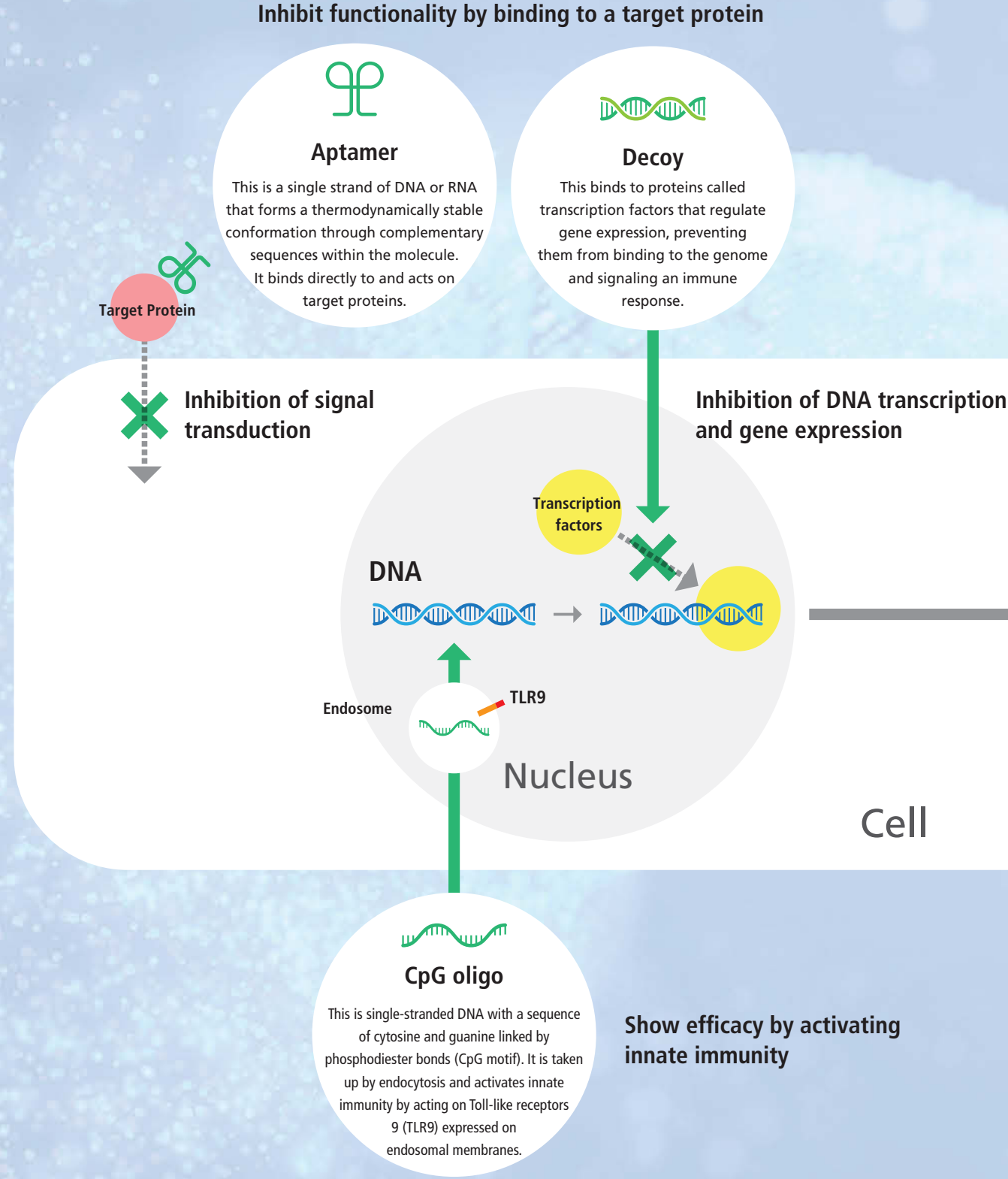
SHIMADZU
Excellence in Science

Oligonucleotide Therapeutics Solution



Types and Characteristics of Olig

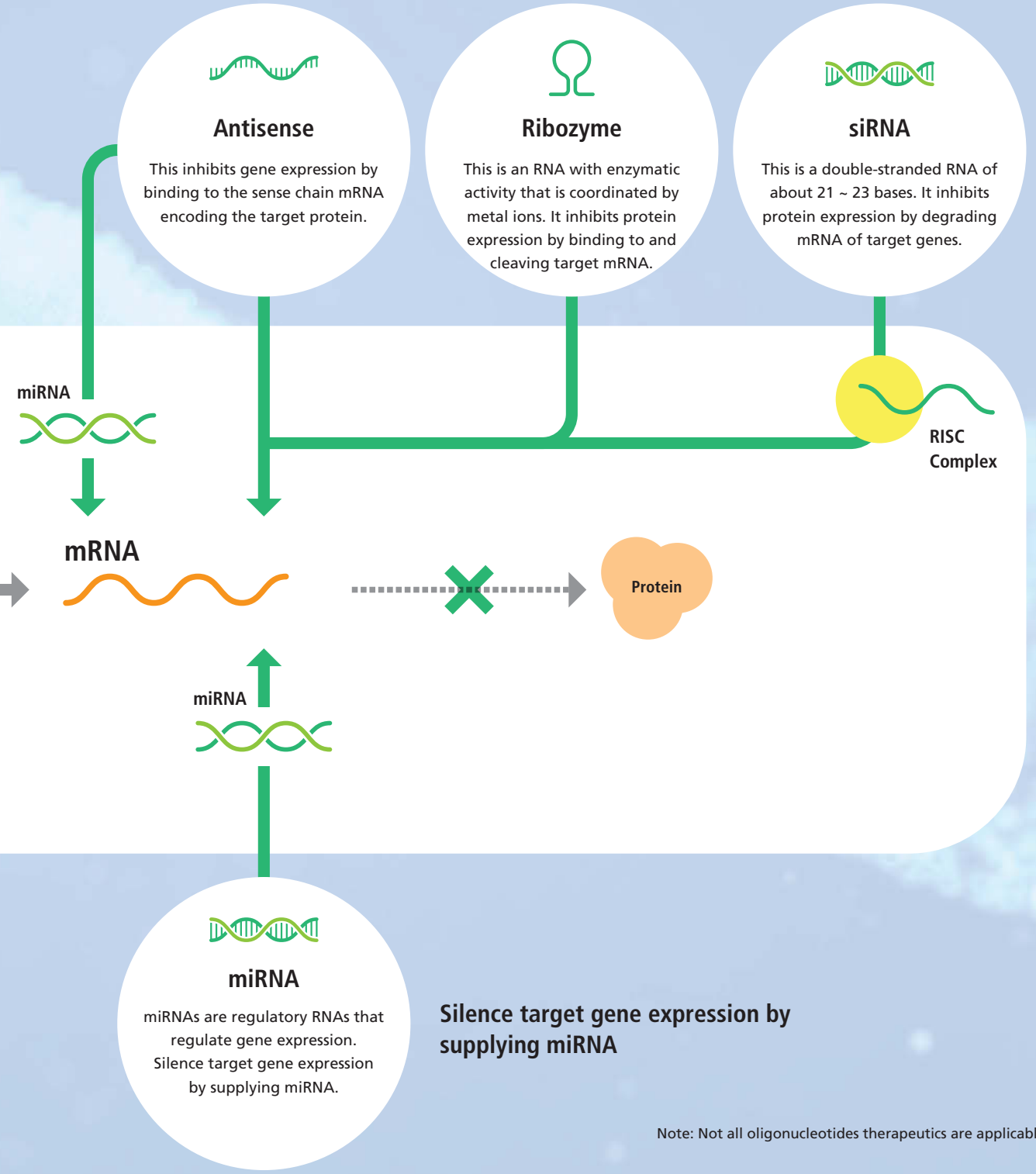
Oligonucleotide therapeutics are comprised of a few to several dozen nucleic acids (including modified ones) and chemically synthesized. Oligonucleotide therapeutics can effectively act on specific diseases and are easy to design and synthesize. These characteristics can faster to find new therapeutic candidates.



gonucleotide Therapeutics

However, oligonucleotide therapeutics are degraded and excreted rapidly after administration by exonucleases and endonucleases that are abundant in blood and cells. This problem is being addressed by the introduction of modified oligonucleotides to improve chemical stability in vivo and by the development of lesion-targeted DDS (drug delivery systems) technologies.

Inhibit gene expression by binding to target RNA



Silence target gene expression by supplying miRNA

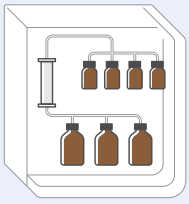
Note: Not all oligonucleotides therapeutics are applicable.

Analytical Instruments Utilizing Synthesis and Purification Processes Optimization

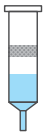
Laboratory Scale

Oligonucleotide Synthesis

Synthesis



Crude purification



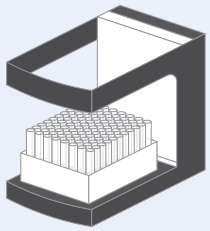
Molecular weight confirmation of products after crude purification

- MALDI-MS
- LC-MS (SQ)

Feedback
Optimization of synthesis parameters

Oligonucleotide Purification

Purification



Nexera Prep

Molecular weight confirmation of target products and impurities in the collected sample

- MALDI-MS
- LC-MS (SQ)

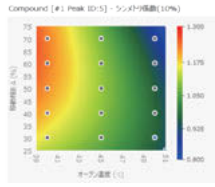
Feedback
Optimization of purification parameters

Characterization and Analysis

Optimization of parameters for separating target products and impurities

- HPLC•LabSolutions MD
- LC-MS (SQ)

LCMS-2050



LabSolutions MD

Molecular weight confirmation and sequencing of target products and impurities

- LC-MS (Q-TOF•SQ)
- MALDI-MS

# of oligonucleotide Therapeutics

Manufacturing process

Analysis test

Scaling Up

Method Development

Melting point measurement of target products

- Tm analysis

Tm Analysis System

Quantitative analysis and spectrum confirmation

- UV

UV-1900i Plus

Development of New Analysis Methods

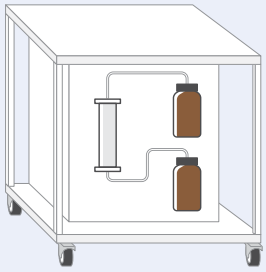
Approaches for difficult samples to separate and analyze using conventional methods

- Supercritical Fluid Chromatography (SFC)

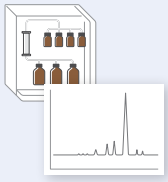
Nexera UC

Synthesis, Crude Purification, and Purification

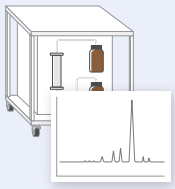
Nucleic acid synthesis and purification



Before scaling up



After scaling up



Evaluation of correlation between before and after scaling up

Molecular weight confirmation of partially purified products

- MALDI-MS
- LC-MS (SQ)

Molecular weight confirmation and sequencing of target products and impurities in the collected matter

- LC-MS (Q-TOF•SQ)
- MALDI-MS

Quality Control

Residual solvent analysis

- GC

Brevis GC-2050

Elemental impurity analysis

- ICP-MS

ICPMS-2050

Pharmacokinetics

Quantitative analysis and molecular weight

- LC-MS (TQ)

LCMS-8060RX

Efficient Method Development of Oligonucleotides Using LabSolutions MD

Application

Step1 Column and Mobile Phase Screening

■ Screening Parameters (Examples)

Separation column	Aqueous mobile phases	Organic solvents
	• Alkylamine (type and concentration) • HFIP concentration	• Methanol/acetonitrile mixture ratio

Automatic generation of analysis schedule

Mobile phase blending function

Column switching function

Continuous analysis under multiple conditions

Automatic search for optimal conditions from acquired data (ranking of chromatograms based on Evaluation Values)

Step2 Optimization of LC Parameters

■ Parameters for Optimization (Examples)

Column oven temperature	Organic solvent composition	Gradient conditions

Automatic generation of analysis schedule

Continuous analysis under multiple conditions

Automatic peak tracking

Optimization of gradient conditions (AI search)

Search for optimal conditions using design space

Analysis under the optimized conditions

Solution for Method Development and Analytical Quality by Design

LabSolutions MD

Product

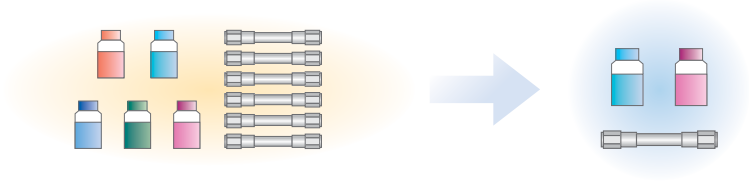
Features

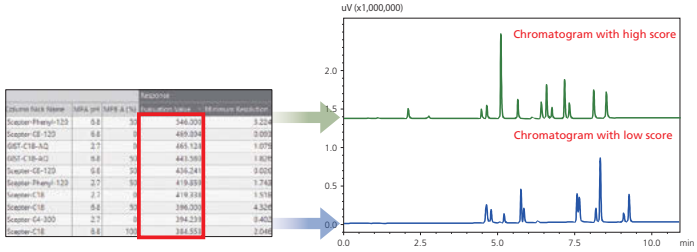
Improving Efficiency of the Entire Method Development Workflow

LabSolutions™ MD improves method development efficiency by taking an Analytical Quality by Design (AQbD) approach.
This software efficiently develops highly reliable analysis methods by configuring mobile phases, columns, and other parameters using an analysis function that automatically generates analysis schedules with the experimental design method and a data analysis function that plots a design space and predicted chromatogram.

Step 1: Initial Screening

Initial screening is based on parameters that have a major effect on peak retention time and separation, such as the pH of aqueous mobile phases, the mixture ratio of organic mobile phases, and the type of column. LabSolutions MD quickly searches vast amounts of data to determine the optimal analysis conditions.



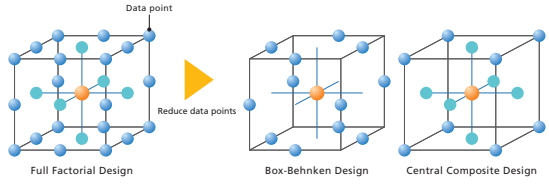


Ranking Based on Each Condition and Evaluation Value

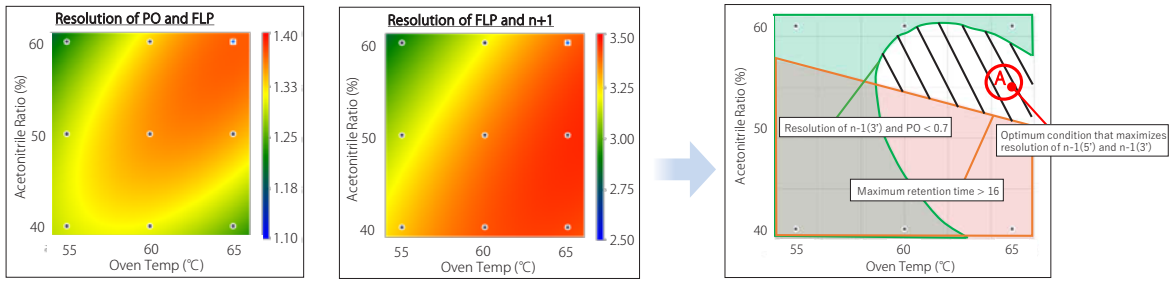
Comparison of Chromatograms with High and Low Evaluation Value

Step 2: Optimization

With the analytical conditions determined by initial screening results as a starting point, optimal setting levels are verified for other parameters, such as pump gradient and column oven temperature conditions.
Experimental design can be used to reduce the number of experiment data points.



The relationship between factors and responses can be visually represented as a design space.



Design Space for Resolution of FLP and Related Impurities

Overlay of Design Spaces of Resolution and Retention Time of Last Eluting Peak

Step 3: Robustness Evaluation

Validation verifies that small variations in the optimized analytical condition settings affect measurement values only within an allowable range.

Software for Oligonucleotide Characterization

LabSolutions Insight Biologics

Product

This data analysis software can be used for every process, from development to quality control, and with a wide variety of mass spectrometers.

Applicable Models

MALDI-MS
MALDI-8030
MALDI-8020
MALDImini-1

LCMS-QTOF
LCMS-9050
LCMS-9030

LCMS-SQ
LCMS-2050
LCMS-2020

Confirmation after Crude Purification	✓	✓	✓
Confirmation after Purification	✓	✓	✓
Quality Control		✓	✓

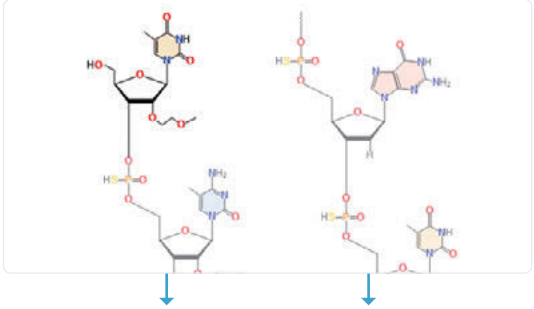
Entering Sequence Information for Oligonucleic Acids in Target Products

- If sequence information is entered, structural formulas are shown visually for easier checking.
- Sequence information for created target products can be saved within data analysis methods. User-specified parameters can be referenced even if using a variety of mass spectrometers (SQ, Q-TOF, or MALDI).

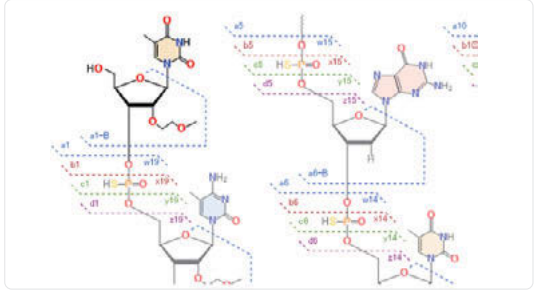
Input oligonucleotide sequences

#	Name	Base	Base Modification	Linker	Ribose	Formula	Mono-isotopic
1	Cd	Cytosine	None	----	Deoxy	C9 H11 N3 O2	193.08513
2	sTd	Thymine	None	Phosphorothioate	Deoxy	C10 H13 N2 O6 P S	320.02319
3	sGd	Guanine	None	Phosphorothioate	Deoxy	C10 H12 N5 O5 P S	345.02968
4	sCd	Cytosine	None	Phosphorothioate	Deoxy	C9 H12 N3 O5 P S	305.02353
5	sTd	Thymine	None	Phosphorothioate	Deoxy	C10 H13 N2 O6 P S	320.02319
6	sAd	Adenine	None	Phosphorothioate	Deoxy	C10 H12 N5 O4 P S	329.03476
7	sGd	Guanine	None	Phosphorothioate	Deoxy	C10 H12 N5 O5 P S	345.02968

The structural formula of sequences is shown in real time



Base modifications can be added and edited

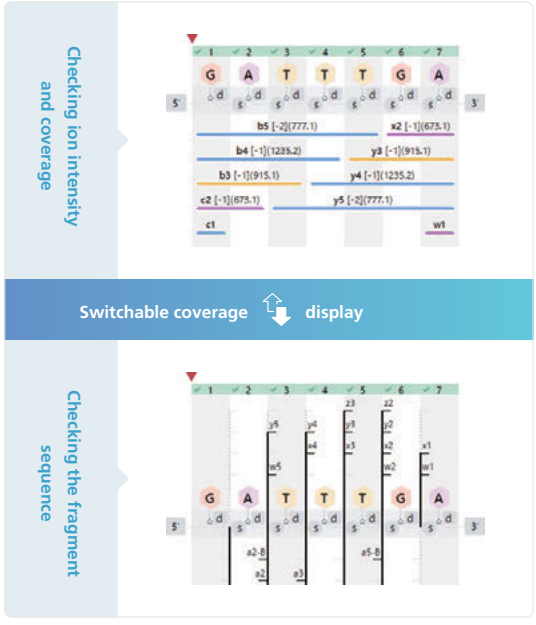


In the window for setting the oligonucleotide sequences, the structural formula of the sequence that was entered is displayed in real time, enabling quick and easy verification of the information. Also, the nucleobases used in the sequence, as well as the backbone linker, ribose, and base modifications, can be added and edited.

Data Analysis of MS Spectra (Simple Molecular Weight Confirmation of Target Products and Impurities)

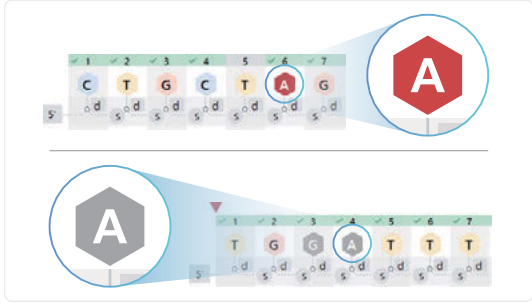
Mass information for specified target products and impurities is automatically identified based on results from analyzing their molecular weight information. Identifying only the principal components requires less than one second.
Only the two LCMS parameter settings specifically required for multi-charged ion analysis of nucleic acids are specified (charge state range and instrument mass accuracy level).

MS/MS Sequence Analysis (Detailed Sequencing of Target Products and Impurities)



The software includes a coverage display which indicates fragment spectral assignments. The coverage display switches to match the items to be checked. Reports can also be output.
The MS/MS DDA mode for automatic fragment acquisition and analysis is used for sequencing.
For data analysis, the software automatically searches all data for precursor ions of components identified by sequencing, which can be expected to increase speed and improve coverage. This means precursor information for each component does not need to be entered manually to configure analysis settings.

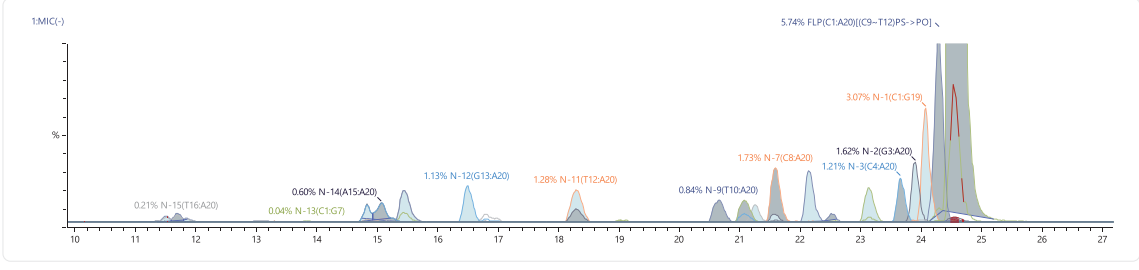
Modification positions are also clearly identified



Component Chromatogram Display

Note: It cannot be used for data acquired with MALDI.

- Existing impurity peaks are easily identifiable in chromatograms.
  Based on measured mass spectra, chromatograms are displayed based on a combination of all charge state differences and isotope spectra. This enables a comprehensive display.
- Both UV and MS chromatograms can be checked at the same time. Component ratios can be calculated based on a combination of UV and MS peak area values.



Oligonucleotide Analysis by Ion Exchange Chromatography (IEX)

Application

benefits

- Short chain oligonucleotides can be separated based on the base unit.
- Oligonucleotides can be separated from impurities, such as protecting groups used in the chemical synthesis process.
- Analysis can be performed using mobile phases with high salt concentrations and a wide pH range.

Methods and Results

Sample	5′-TCTTGGTTACATGAAA-3′ (16 mer) 5′-TCTTGGTTACATGAAAT-3′ (17 mer) 5′-TCTTGGTTACATGAAATC-3′ (18 mer) 5′-TCTTGGTTACATGAAATCC-3′ (19 mer) 5′-TCTTGGTTACATGAAATCCC-3′ (20 mer)
Conc., Volume	5 µmol/L, 4 µL
Preparation	Dilution in ultrapure water to the concentrations above.
Analytical Conditions	As shown in Table 1
Results	Target oligonucleotides in 20 mer and 4 sequences that were deleted from n-1 to n-4 on the 3′ terminus of target were prepared as impurities derived from the synthesis. All of them were unmodified single-stranded DNA and synthesized by a solid phase synthesis (HPLC-purified). Figure 1 shows a chromatogram of a mixture of five-sequence oligonucleotide. Each oligonucleotide was separated by their length. Table 2 shows the relative standard deviations (% RSD, n = 6) of the retention time and area of the 16 - 20 mer oligonucleotide mixture, with RSD% less than 1% for both parameters. Then, a mixture of five oligonucleotides was prepared (four of them were HPLC-purified while 1 was only desalted) and compared with the mixture of all HPLC-purified nucleotides (Figure 2). The target oligonucleotides were completely separated from impurities, such as free protecting groups and shorter length oligonucleotides.

Table 1 Analysis Conditions

System :	Nexera XS inert
Column :	Shim-pack Bio IEX Q-NP (100 mm × 4.6 mm I.D., 5 µm)
Mobile phase A :	10 mmol/L NaOH
Mobile phase B :	10 mmol/L NaOH containing 1 mol/L $NaClO_4$
Flow rate :	0.8 mL/min
Time program : (B Conc.)	25-32.5% (0-15 min) → 100% (15-20 min) → 25% (20-25 min)
Column temp. :	30 °C
Injection volume :	4 µL
Detection :	UV 260 nm (SPD-M40), UHPLC standard cell
Vial :	Shimadzu 1.1 mL sample vial

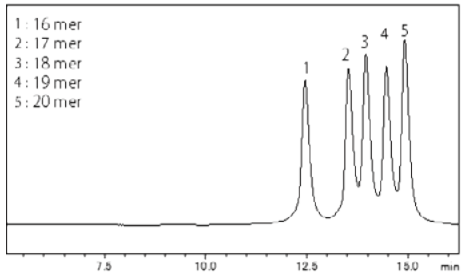


Figure 1 Chromatogram of oligonucleotides mixture

Table 2 Relative standard deviation (% RSD) of each component (n = 6)

Length(mer)	Retention time	Area
16	0.138	0.224
17	0.105	0.335
18	0.098	0.494
19	0.085	0.161
20	0.075	0.307

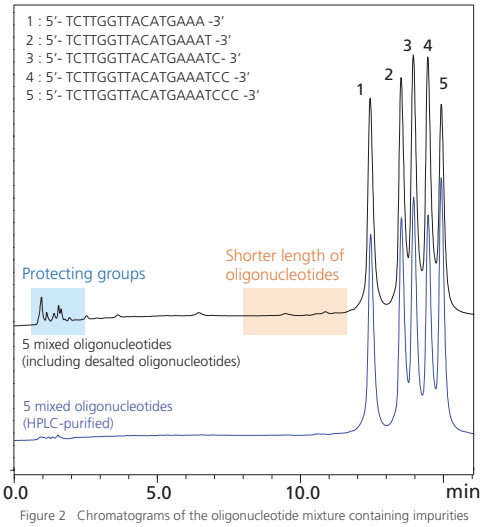


Figure 2 Chromatograms of the oligonucleotide mixture containing impurities

Conclusions

By using Nexera XS inert and Shim-pack Bio IEX, it is possible to reproducibly separate the desired oligonucleotide from impurities such as protecting groups generated during chemical synthesis or oligonucleotides with different chain lengths generated by incomplete synthesis.

Nexera XS inert

Product

Features

The potential adsorption of an analyte onto wetted surfaces of UHPLC instruments poses some critical challenges when analyzing biomolecules. While elevated pressure tolerance is required to achieve optimal chromatographic separation when using small particle size columns, the inertness of the wetted surfaces is also of the utmost importance, as is resistance to corrosion due to the use of mobile phases with high salt concentrations and extreme pH values.
The Nexera XS inert system offers the ideal solution for the separation of biomolecules by combining the elevated pressure tolerance of a UHPLC system with complete inertness of the sample flow path, ensured by the absence of wetted metal surfaces and ultra-high resistance to corrosion.

System Controller
SCL-40, CBM-40/40lite
Used to coordinate actions of the overall system, this controller offers intuitive operation that minimizes the stress involved in operating the system, from startup to shutdown.

Detector
SPD-40/40V/M40
Inert type cells for UHPLC analysis eliminate the risk of adsorption on the detector. A low-diffusion cell, which has 5 mm of optical path length, can also be selected.

Solvent Delivery Unit
LC-40D XSi
Designed with corrosion-resistant non-stainless steel materials, it offers rugged, low-pulsation performance, advanced AI features and solvent blending capabilities. (optional).

Autosampler
SIL-40C XSi
This high-performance autosampler features nonmetal materials for all surfaces that contact liquids. That inhibits metal-adsorption of biomolecules.

UHPLC Inert Switching Valve
FCV-0206H2i/FCV-0607H2i
Designed with adsorption-inhibiting materials for all wetted surfaces.

EXPERIENCE NEWFOUND CLARITY

Unconstrained Recovery and Sensitivity

Reduces sample loss due to adsorption to metal surfaces and achieves excellent sensitivity.

Clear Resolution without Restrictions

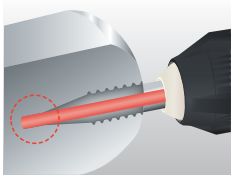
Improves peak shape and achieves excellent chromatographic separation.

Assured Reliability and Reproducibility

Reliable data for metal-adsorbing compounds with high reproducibility.

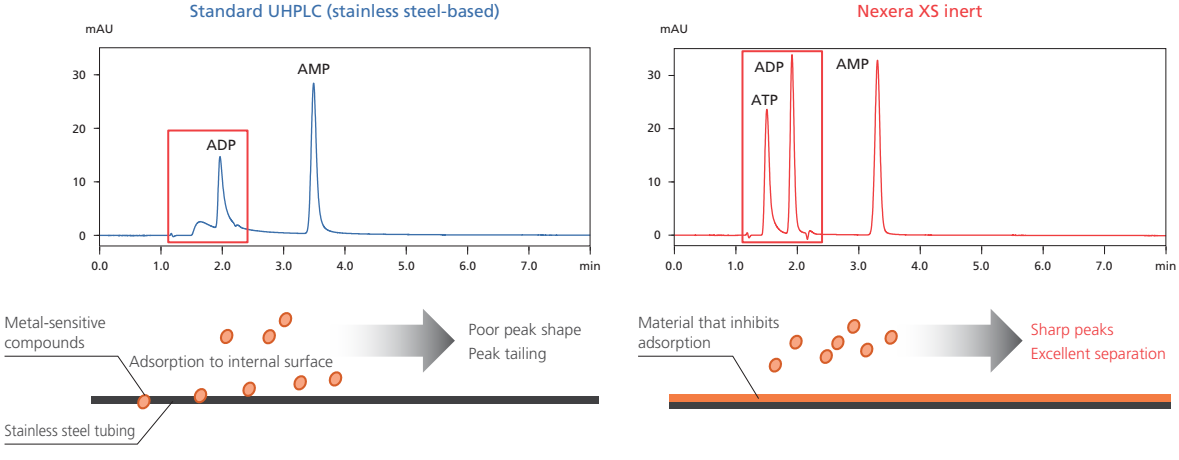
Finger Tight Fittings for Simple and Secure Connections

Nexera XS inert systems feature tubing connections with unique finger-tight fittings. They can achieve connections with up to 105 MPa of pressure capacity without creating any dead volume.



Resolution without Restrictions

The Nexera XS inert system is equipped with unique technology that ensures the complete inertness of the sample flow path. The system provides excellent peak shape and unsurpassed chromatographic separation by effectively inhibiting the adsorption of target compounds to internal surfaces.



Improvement of Oligonucleotide Peak Shape Using Automatic Pretreatment Function (Co-injection)

Application

benefits

- Sample dilution can be automated using the automatic pretreatment function "co-injection" for purified fractions of oligonucleotides containing high concentrations of salts.
- The automatic pretreatment function "co-injection" enables the simultaneous injection of a sample and a selected co-injection solvent. The co-injection solvent and its injection volume can be easily set via LabSolutions MD interface.

Introduction

In HPLC analysis, the composition of the sample solvent is critical for achieving proper peak shape. If the sample solvent is a stronger eluting solvent than the mobile phase, sample band condensation at the column inlet may be insufficient, leading to sample band broadening. For instance, increasing the organic solvent ratio in the sample solvent to dissolve low-polar compounds may deteriorate the peak shapes of early-eluting compounds in reversed-phase chromatography. Oligonucleotides are synthesized using the phosphoramidite method and subsequently purified by anion exchange chromatography or reversed-phase ion-pair chromatography (RPIP). Purified fractions from anion exchange chromatography often contain high concentrations of salts, such as sodium chloride (NaCl) and sodium bromide (NaBr). When these purified fractions are analyzed using RP-IP, the high salt concentration in the sample solvent may interfere with the ion-pair formation capacity of the oligonucleotides. This interference can result in improper peak retention and adversely affect the chromatogram.

Analytical Conditions

The analytical conditions are shown in Table 1. Two types of salts, 3 mol/L NaCl and 3 mol/L NaBr, were added to the sample solvent to simulate purified fractions containing high concentrations of salts. To assess the effects of co-solvents and co-injection volumes on peak shape, four different co-solvents were used : water and three solutions containing HA (hexylamine) at concentrations of 10, 30, and 50 mmol/L, each prepared in 100 mmol/L HFIP (1,1,1,3,3,3-hexafluoro-2-propanol). Additionally, three injection volumes (1, 3, and 5 µL) were evaluated. By simply entering the vial numbers and injection volumes of the co-injection solvents via the LabSolutions MD (red frame in Figure 2), the autosampler automatically adjusts the co-injection solvent types and volumes during analysis. This enables automated comprehensive evaluation of conditions, greatly reducing manual labor.

"Co-injection" Function

The co-injection function, equipped in autosamplers, provides automatic pretreatment. A specified amount of reagent or solvent from any vial can be mixed with the sample solution in the needle and injected (Figure 1).

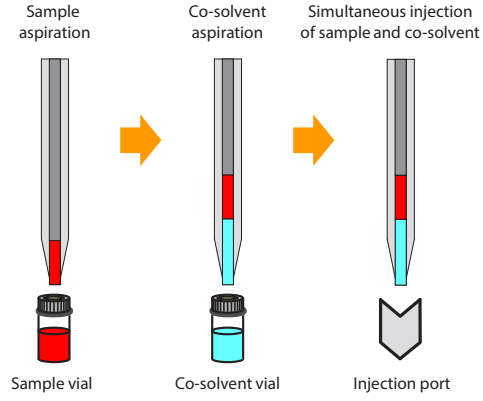


Figure 1 Flow Diagram of Co-injection Sequence

			Pretreatment Program	
Use	Sample Name	Vial	co-solvents vial	co-injection volume(µL)
☑	Oligonucleotide 1	1	2	1
☑	Oligonucleotide 2	1	2	3
☑	Oligonucleotide 3	1	2	5
☑	Oligonucleotide 4	1	3	1
☑	Oligonucleotide 5	1	3	3
☑	Oligonucleotide 6	1	3	5
☑	Oligonucleotide 7	1	4	1
☑	Oligonucleotide 8	1	4	3
☑	Oligonucleotide 9	1	4	5
☑	Oligonucleotide 10	1	5	1
☑	Oligonucleotide 11	1	5	3
☑	Oligonucleotide 12	1	5	5

Figure 2 Setting Screen of co-injection (LabSolutions MD)
Vial 2 : Co-solvent 1, Vial 3 : Co-solvent 2, Vial 4 : Co-solvent 3, Vial 5 : Co-solvent 4

Methods and Results

Table 1 Analysis Conditions

System :	Nexera XS inert (Method Scouting System)
Column :	Accura Triart Bio C18[*1] (100 mm × 2.1 mm I.D., 1.9 µm)
Sample solvent 1 :	20 mM Tris-HCl (pH 8.5) containing 3 mol/L NaCl
Co-solvent 1 :	Water
Co-solvent 2 :	100 mmol/L HFIP[*2] and 10 mmol/L HA[*3] in water
Co-solvent 3 :	100 mmol/L HFIP and 30 mmol/L HA in water
Co-solvent 4 :	100 mmol/L HFIP and 50 mmol/L HA in water
Co-injection volumes :	1, 3, 5 µL
Mobile phases	
Pump A :	100 mmol/L HFIP and 10 mmol/L HA in water
Pump B :	100 mmol/L HFIP and 10 mmol/L HA in Methanol
Temperature :	60 °C
Injection volume :	1 µL
Flow rate :	0.2 mL/min
Time program (%B) :	25 % (0-2 min) → 67 % (26 min) → 100 % (26-27 min) → 25 % (27-35 min)
Detection :	260 nm (SPD-M40, UHPLC inert cell)

*1 YMC.CO., LTD.
*2 1,1,1,3,3,3-hexafluoro-2-propanol
*3 Hexylamine

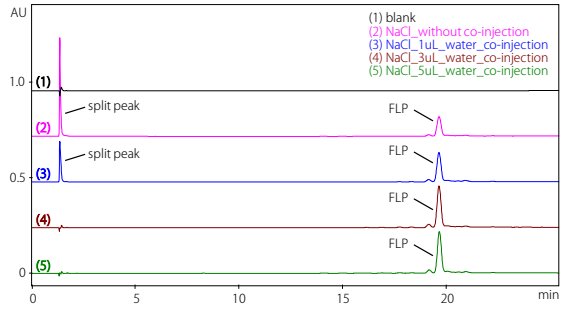


Figure 3 Comparison of Chromatograms with/without Co-injection
(sample solvent : NaCl)

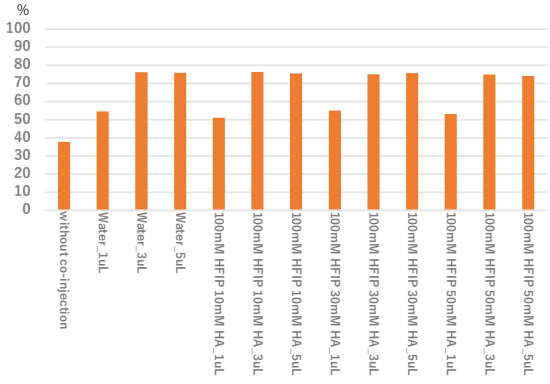


Figure 4 Normalized FLP Peak Areas (sample solvent : NaCl)
* normalization based on sum of all peaks including FLP related impurities

Chromatograms obtained without the co-injection function are shown in Figure 3 (sample solvent : NaCl). The ion-pair formation ability was suppressed due to the high concentration of salts in the sample solvents. Consequently, the oligonucleotide was not properly retained, and part of the peak was eluted as a split peak. On the other hand, simultaneous co-injection of pure water resulted in increased peak areas for the full-length product (FLP), confirming that it effectively provided appropriate peak retention. When the co-injection volume was 3 µL or more (Figure 3 (4), (5)), split peaks were no longer observed. The relationship between the co-solvents, co-injection volumes, and the normalized peak areas (%) of FLP is shown in Figure 4 (sample solvent : NaCl). Regardless of the co-injected solvent type, the normalized peak areas of FLP remained nearly constant when the co-injection volume was 3 µL or more. This suggests that co-injection is effective in suppressing the peak splitting phenomenon caused by the sample solvent effect.

Conclusion

When purified fractions of oligonucleotides containing high concentrations of salt are analyzed by RP-IP, the ion-pair formation capacity is suppressed, and peaks may not be properly retained, resulting in partial early elution as a split peak. Pretreatment, such as desalination or dilution of samples, is usually required before analysis, but it is time-consuming. The co injection function, equipped in Nexera series autosamplers, allows for the simultaneous injection of a desired solvent with purified fractions, providing automatic pretreatment that enables the direct analysis of oligonucleotides in purified fractions with high salt concentrations. In particular, when many purified fractions need to be analyzed, significant labor savings can be achieved because the necessary pretreatment procedures are automated for all fractions. The optimal co-injection solvent and volume depend on the analytical conditions, such as oligonucleotide sequence and mobile phase composition. Therefore, although optimization of conditions is required each time, the use of LabSolutions MD enables efficient comprehensive evaluation of conditions.

<Acknowledgments>
A part of this work was supported by AMED under Grant Number JP21ae0121022, JP21ae0121023, JP21ae0121024 (Project leader: Satoshi Obika).

Confirming Molecular Weight of 70-mer Synthetic DNA Sequences Using a Single Quadrupole Mass Spectrometer

Application

benefits

- Oligonucleotides can be easily analyzed using a Nexera XS inert UHPLC system and an LCMS-2050 single quadrupole mass spectrometer.
- The molecular weight of oligonucleotides can be estimated by deconvoluting the obtained mass spectra.

Methods and Results

Sample	Single-stranded synthetic DNA GGTGTCAGGCTCACGGACCACTGCACAACAATCCCACGACGT CGCCATTTTCTGCGATCCGGCAAGGCGA
Concentration and Quantity	10 pmol
Analysis	The above oligonucleotide was analyzed using the analysis conditions listed in Table 1.
Results	The total ion chromatogram obtained from analyzing the synthetic DNA is shown in Figure 1. Based on the mass spectrum (Figure 2) of the peak detected near a retention time of 11 minutes, multi-charged ions with charge states from 17 to 22 were detected. Deconvolution analysis for the multi-charged ions resulted in calculating an estimated molecular weight of 21465.4 (Figure 3) and a theoretical molecular weight of 21465.9 with a small mass error level of 1 Da or less.

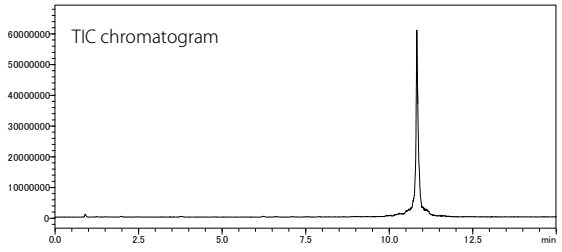


Figure 1 TIC Chromatograms of the Synthetic DNA

Table 1 Analysis Conditions

[HPLC conditions] (Nexera XS inert)	
Column :	Shim-pack Scepter Claris C18-120 (100 mm x 2.1 mm I.D., 1.9 µm)
Flow rate :	0.3 mL/min
Mobile phase A :	95.4 mM HFIP, 7.1 mM TEA in water
Mobile phase B :	95.4 mM HFIP, 7.1 mM TEA in methanol
Time program :	5 %B (0-2 min) - 35 %B (15 min) - 80 %B (16-17 min) - 5 %B (18-25 min)
Column temp. :	50 °C
Detection :	PDA 200-400 nm
Injection volume :	2.33 µL (10 pmol)
[MS conditions] (LCMS-2050)	
Ionization :	ESI/APCI (DUIS), Negative mode
Interface voltage :	-2.0 kV
Mode :	Scan (*m/z* 600-2000)
Nebulizing gas flow :	2.0 L/min
Drying gas flow :	5.0 L/min
Heating gas flow :	7.0 L/min
Desolvation temp. :	450 °C
DL temp. :	200 °C

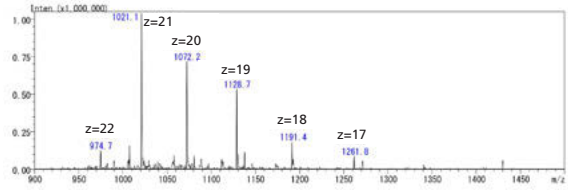


Figure 2 Mass Spectrum of the Synthetic DNA

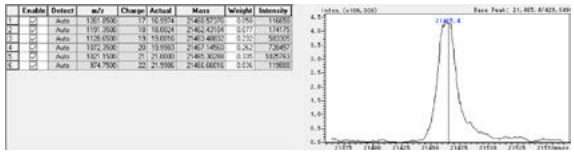


Figure 3 Deconvoluted Mass Spectrum

Conclusion

Generally, when estimating molecular weight using a deconvolution function, the more multiply-charged ions that can be obtained, the more reliable the molecular weight estimation will be. As a result of deconvolution of the mass spectrum for the detected peaks, it was possible to estimate the molecular weight of the principal components of the oligonucleotide therapeutic within a 1 Da margin of error from the theoretical value. The LCMS-2050 enables fast and highly sensitive analysis across a wide mass range, while maintaining user-friendly operation similar to LC systems. The system described above provides a useful analytical tool for quality control of oligonucleotide therapeutics.

<Acknowledgments>
We are grateful to Dr. Yuuya Kasahara (National Institutes of Biomedical Innovation, Health and Nutrition, Japan) for generously providing the sample.

Liquid Chromatograph Mass Spectrometer

LCMS-2050

Features

The LCMS-2050 single quadrupole mass spectrometer system offers a combination of easy usability, high basic performance, and compact size, which makes it easy to use as an LC detector while also ensuring the high analytical capabilities of a mass spectrometer.
Equipped standard with DUIS heated ionization, it provides the benefits of both ESI and APCI injection methods. The LCMS-2050 can measure a wide range of target compounds, from small to large molecules and from low to high polarity levels, making it suitable for a wide variety of applications and fields.

LCMS-2050 with Nexera XS inert

Product

Seamless Connectivity with Shimadzu LC Systems

The small footprint of the LCMS-2050 creates the flexibility to adapt to different needs. As with other LC detectors, It can be integrated into any Shimadzu LC architecture whether it is a high-throughput analytical system, a preparative LC with fraction collection, or even a legacy model.

Easy Parameter Settings for Seamless Operation

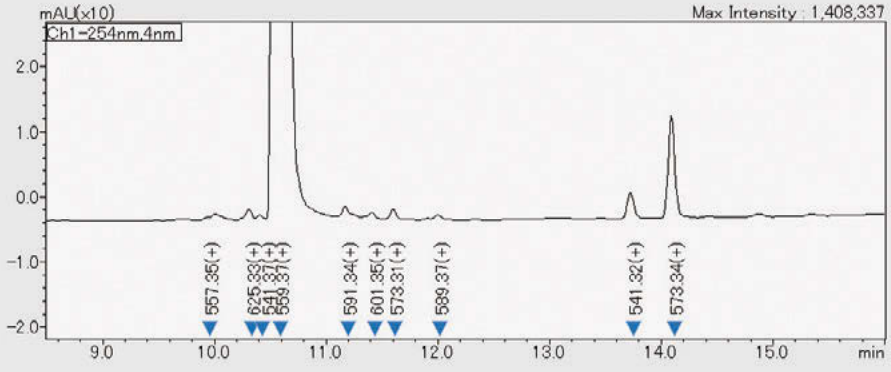
The LCMS-2050 enables analysis by configuring only simple parameter settings similar to a PDA detector. It can perform everything from MS data acquisition to data analysis with an operating feel that is no different than using an LC unit.

Adding MS to Complement and Confirm Optical Detection

UV-PDA and MS are orthogonal technologies each having their own strengths. MS detectors are generally more sensitive than optical detectors and, moreover, enable selective detection of compounds by their masses. For this reason, MS detectors can detect co-eluting compounds as well as many analytes which do not respond to UV detection. The LCMS-2050 is a valuable addition to any LC system to help increase reporting confidence.

Mass-it (Mass signature impartment technology)

Mass-it is a new invention for displaying mass information that helps scientists grasp complex data at a single glance. It detects and overlays signature mass information onto the LC-UV or the LC-PDA chromatogram. This makes it possible to spot where there are multiple components co-eluting in a single UV peak and where there are 'hidden' components without UV absorbance. All in a single click, at a single glance. In addition, Mass-it includes functionality for suggesting when multiple components with different masses are coeluted. This reduces the risk of overlooking unknown impurities that are difficult to separate.



UV Chromatogram for Atorvastatin

Molecular Weight Measurement of Drug Substances and Impurities

Simple Analysis of Impurities in Oligonucleotide Therapeutics Using a Single Quadrupole Mass Spectrometer

Application

benefits

- The molecular weights of oligonucleotides and related impurities can be confirmed using an Nexera XS inert LC system and an LCMS-2050 single quadrupole mass spectrometer.
- The LCMS-2050 can be used for simple analysis in the same manner as an LC detector.

Methods and Results

Sample	• FLP (20-mer sequence with theoretical molecular weight of 7169) T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC- dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC* • n-1 (3′) 19-mer sequence with theoretical molecular weight of 6776) T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC- dA-dT-dG-dA-dA-A*-T*-mC*-mC* • n-3 (3′) 17-mer sequence with theoretical molecular weight of 5987) T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC- dA-dT-dG-dA-dA-A*-T* • n-10 (5′) 10-mer sequence with theoretical molecular weight of 3553) dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC* * = 2′-O-methoxyethyl, m = 5-methyl, d = 2′-deoxy
Concentration and Quantity	FLP: 0.17 pmol/µL and 6 µL (equivalent to 1 pmol)
Analysis	A sample prepared by mixing the four types of oligonucleotides indicated above (including deletion variants equivalent to 5 % of FLP) was analyzed using the measurement conditions in Table 1.
Results	LabSolutions Insight Biologics software was used for data analysis. The UV (260 nm) and component chromatograms obtained from analyzing a model oligonucleotide measurement sample are shown in Figure 1. The component chromatogram was obtained by combining the spectra for different isotopes and all charge states based on MS1 spectra. Peaks were confirmed for deletion variants n-10 (5′), n-3 (3′), and n-1 (3′), and FLP, in that order. Mass spectra for peaks derived from FLP and deletion variant n-1 (3′) are shown in Figure 2. Multi-charged ions with 3 to 10 charges were detected. Each mass spectrum was analyzed by multi-charged ion analysis to estimate theoretical molecular weights (Figure 3). This resulted in calculating a molecular weight of 7168 for FLP (7169 theoretical) and 6775 for n-1 (3′) (6776 theoretical), which indicated peaks were identified with a mass error level of 1 Da or less from theoretical values.

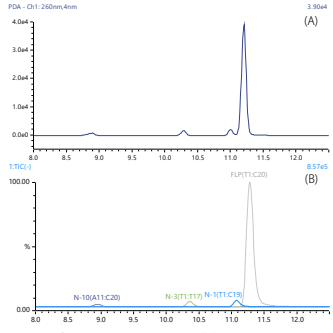


Figure 1 Chromatograms of Model Oligonucleotide (A) UV Chromatogram, (B) TIC Chromatogram

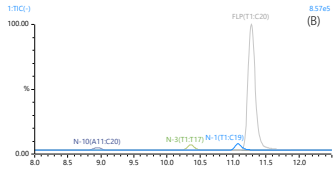


Figure 2 Component Chromatogram from Model Oligonucleotide Analysis

Table 1 Measurement Conditions

[HPLC conditions] (Nexera XS inert)	
Column :	Shim-pack Scepter Claris C18-300 (100 mm x 2.1 mm I.D., 1.9 µm)
Flow rate :	0.3 mL/min
Mobile phase A :	100 mmol/L HFIP, 10 mmol/L TEA in water
Mobile phase B :	Methanol
Time program :	10 %B (0 min) → 35 %B (15 min) → 40 %B (20 min) → 90 %B (20.1-22 min) →10 %B (22.1-26 min)
Column temp. :	60 °C
UV detection :	190-400 nm
Injection volume :	6 µL
[MS conditions] (LCMS-2050)	
Ionization :	ESI/APCI (DUIS), Negative mode
Interface voltage :	-3.0 kV
Mode :	Scan (*m/z* 550-2000)
Nebulizing gas flow :	3.0 L/min
Drying gas flow :	5.0 L/min
Heating gas flow :	7.0 L/min
Desolvation temp. :	450 °C
DL temp. :	200 °C

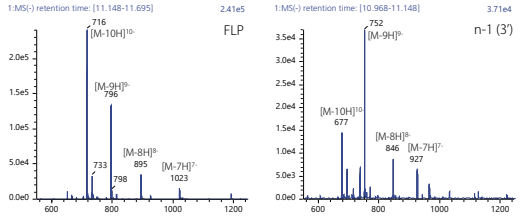


Figure 3 Mass Spectra for FLP and n-1 (3′)

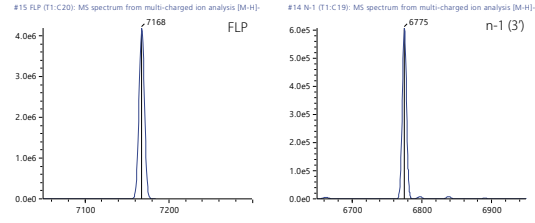


Figure 4 Deconvolved Mass Spectra

Conclusion

The LCMS-2050 single quadrupole mass spectrometer was used to analyze the principal components and related impurities in model oligonucleotide samples. Combining easy operation with excellent performance features makes the LCMS-2050 a useful tool for quality control of oligonucleotide therapeutics.

Liquid Chromatograph Mass Spectrometer

LCMS-2050

Features

Wide Mass Range

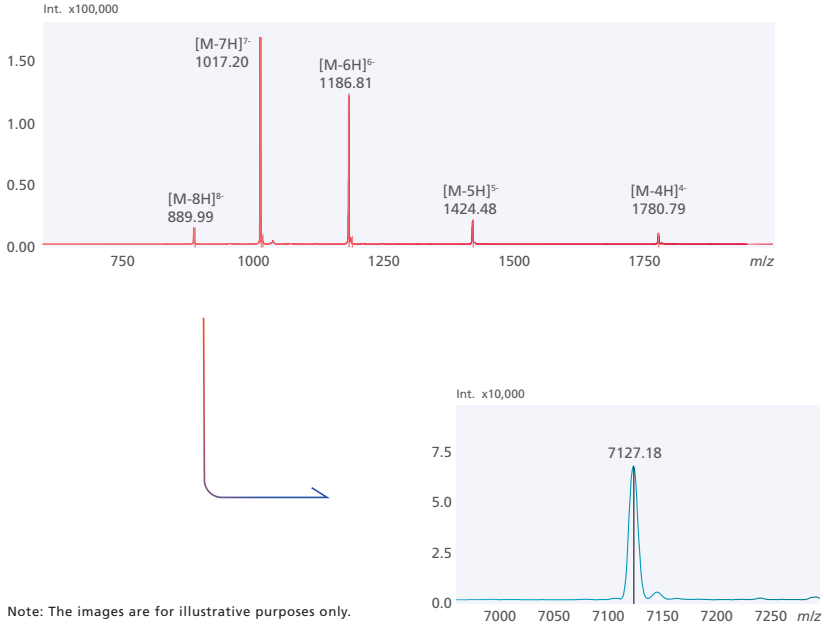
With a wide mass range from *m/z* 2 to 2,000, the LCMS-2050 can be used for a broad range of applications. By using LabSolutions LCMS functionality for multi-charged ion analysis, the molecular weights of large molecules can also be estimated.

Nusinersen Mass Spectrum and Deconvolution Results

When the LCMS-2050 was used to measure nusinersen (theoretical molecular weight: 7127.23), ions with 4 to 8 charges were detected. The molecular weight calculated using the multi-charged ion analysis functionality was an extremely accurate 7127.18, which differs from the theoretical molecular weight by only 0.05 Da. In general, when estimating molecular weight values using multi-charged ion analysis functionality, the more multi-charged ions detected, the higher the reliability of estimated molecular weight values. The broad mass range of the LCMS-2050 means the system can also be used to analyze not only nucleic acids, but also peptides, proteins, polymers, and other large molecules.

LCMS-2050

Product



Note: The images are for illustrative purposes only.

LabSolutions Insight Biologics Oligonucleotide Characterization Software Enables Data Analysis

LabSolutions Insight Biologics can be used to easily identify target substances and impurities by selecting a nucleic-acid base, linker, ribose, modification, or other structure and creating a sequence.

Analysis of Oligonucleotide Impurities Using Single Quadrupole Mass Spectrometer

Application

benefits

- Combination use of LCMS-2050 single quadrupole mass spectrometer and LabSolutions Insight Biologics provides comprehensive characterization of oligonucleotides and related impurities.
- Purity calculations for target components can be performed even on poorly separated peaks by utilizing MS spectra simultaneously acquired with UV chromatogram.

Introduction

In quality control process, the use of a single quadrupole mass spectrometer is expected to be advantageous because of providing mass information with easy operation when impurity information is already known. This article presents a simulated impurity analysis of synthetic oligonucleotide using the LCMS-2050 high-performance liquid chromatography-mass spectrometer and LabSolutions Insight Biologics.

Analytical Conditions

The employed instrument setup was Nexera XS inert and LCMS-2050. HPLC conditions are shown in Table 2, and MS conditions are shown in Table 3. The conditions were set where n-1(5') co-eluted with FLP, while n-3(5') was separated (Figure 1) to demonstrate impurity analysis performance.

Table 1 Sequence information for respective components*

Abbreviations	Sequence (5′ → 3′)
FLP	MG-MC-MC-MU-MC-dA-dG-dT-dT-dC-dT-dG-dC-dT-dC-MG-MC-MA-MC-MC
n-1(5′)	MC-MC-MU-MC-dA-dG-dT-dC-dC-dT-dG-dC-dT-dC-MG-MC-MA-MC-MC
n-3(5′)	MU-MC-dA-dG-dT-dC-dT dG-dC-dT-dT-dT-dC-MG-MC-MA-MC-MC

* Positions 5 of M: 2′-O-(2-methoxyethyl) nucleoside; d: 2′-deoxynucleoside; C: cytosine, and U: uracil are substituted by methyl groups. All phosphodiester bonds between nucleotides have been substituted by phosphorothioate bonds.

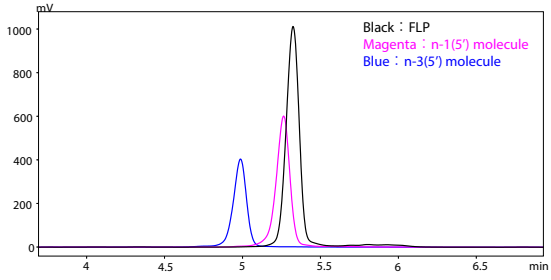


Figure 1 Chromatograms of FLP, n-1(5′), and n-3(5′) (260 nm by PDA detector)

Table 2 HPLC analytical conditions

System :	Nexera XS inert
Column :	Shim-pack Scepter Claris C18-120 (100 mm × 2.1 mm I.D., 1.9 µm)*1
Mobile Phase A :	100 mmol/L HFIP, 10 mmol/L TEA in water
Mobile Phase B :	100 mmol/L HFIP, 10 mmol/L TEA in methanol
Time Program :	15 % B (0 min) → 30 % B (6 min) → 45 % B (6.1-8 min) → 15 % B (8.1-11 min)
Flow Rate :	0.4 mL/min
Column temp. :	60 °C
Injection volume :	5 µL
UV Detection :	190-800 nm (photodiode array detector)

*1 P/N : 227-31210-02

Table 3 MS detection conditions

Ionization :	ESI/APCI (DUIS), Negative mode
Mode :	Scan (*m/z* 650-2000)
Nebulizing gas flow :	3.0 L/min
Drying gas flow :	5.0 L/min
Heating gas flow :	7.0 L/min
Desolvation temp. :	500 °C
DL temp. :	250 °C
Interface voltage :	-3.0 kV

Data processing parameters

In LC-MS, even an isolated single component may be detected at different m/z due to differences in valence or isotopes. Insight Biologics can combine peaks originated from different valences or isotopes into a single component chromatogram to be displayed. In this article, the peak areas of respective components are calculated using the following formula.

$$\text{Component peak area} = (\text{peak area in UV chromatogram}) \times \left(\frac{\text{MS peak area of target component involved in UV–corresponding peak}}{\text{Sum of MS peak areas of all components involved in UV–corresponding peak}} \right)$$

Quantitation od impurities in FLP

The linearity of the component peak area and concentration of FLP was confirmed using LabSolutions Insight Biologics. The results showed a coefficient of determination of 0.999 or higher over the range of 1, 5, 10, 25, and 50 µmol/L. Next, samples were prepared and analyzed by adding each nucleotide-deficient to FLP at 20 µmol/L, which was within the linearity range. Both nucleotides-deficient of n-1(5') and n-3(5') were detected and identified when added at 0.25% or more relative to FLP (equivalent to an added concentration of 0.05 µmol/L). Figure 2 shows the UV chromatogram and component chromatogram for n-1(5') added at 0.25% relative to FLP. Figure 3 and 4 show the results of multivalent ion data processing.
The concentrations of each nucleotide-deficient contained in FLP and obtained component peak areas showed good linearity with a contribution rate of 0.999 or higher over the above addition concentration range (0.25, 0.5, 1, 2, 5%) as shown in Figure 5.

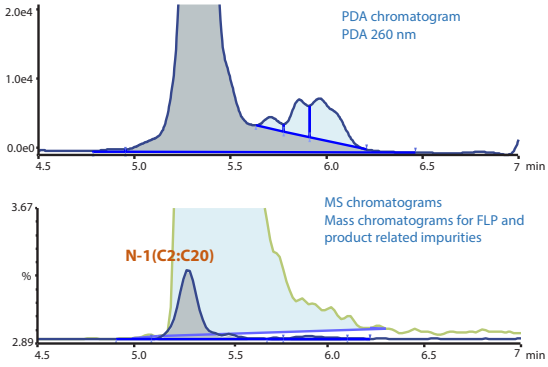


Figure 2 Chromatograms of FLP containing 0.25% of n-1(5')

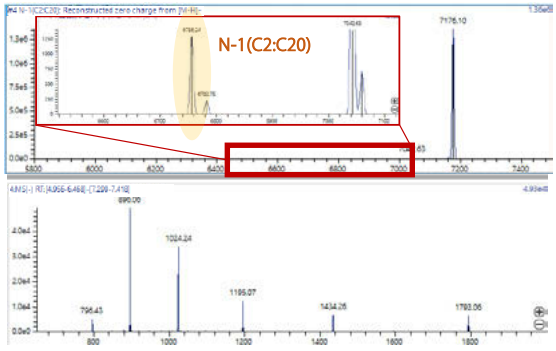


Figure 3 Result of multivalent ion data processing for FLP containing 0.25% of n-1(5') (n-1(5') shown in Figure 2)
Upper : MS spectrum of multivalent ion data processing
Lower : MS spectrum

#	Peak	RT	Area	Mass	Mass Error (ppm)	Mass Error (mDa)	Multi-charge m/z(s)
2	N-1(G1:C19)	4.888	65	6782.43	-87.599	-594.14	846.96,967.75,1129.42
4	N-1(C2:C20)	5.338	16782	6756.24	-117.384	-793.07	749.80,843.57,964.16,1125.16,1349.81

Figure 4 Result of multivalent ion data processing for FLP containing 0.25% of n-1(5') Identification result

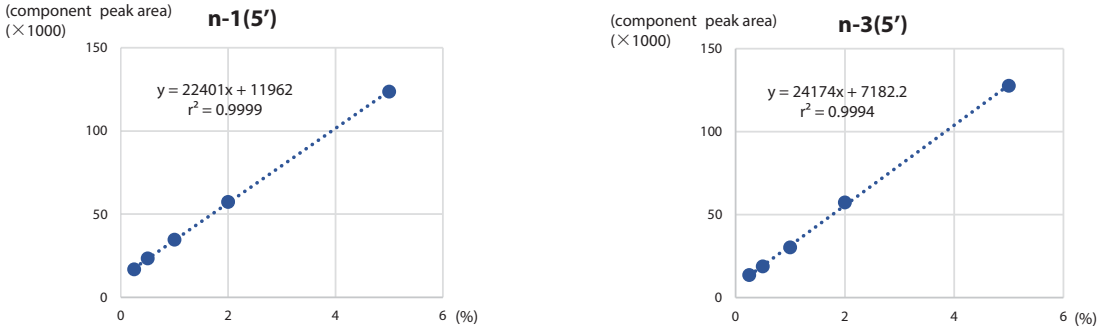


Figure 5 Linearity evaluation for additional concentration

Conclusion

The simulated sample consisting of mainly FLP and small amounts of n-1(5')/n-3(5') was analyzed using the LCMS-2050 and LabSolutions Insight Biologics software and obtained data were processed appropriately. Detection, identification, and quantitation at low concentrations were accomplished not only for n-3(5') well-separated from FLP but also for n-1(5') co-eluted with FLP. In case that establishing analytical conditions is difficult, Using this instrument setup and software will provide efficient quality confirmation of oligonucleotide therapeutics.

<Acknowledgments>
A part of this work was supported by AMED under Grant Number JP21ae0121022, JP21ae0121023, JP21ae0121024 (Project leader: Satoshi Obika).

Efficient optimization to separate oligonucleotides by Reversed-Phase Ion-Pair Chromatography

Application

benefits

- Using LabSolutions MD software can significantly reduce the labor requirements for developing LC methods, such as for screening mobile phases or evaluating various parameters.
- An AI algorithm can optimize gradient conditions, regardless of the operator skill level.
- Useful for evaluating optimal conditions for separating oligonucleotide solutions that include impurities, such as sequences with different chain lengths.

Methods and Results

Sample	Synthetic oligonucleotides with a series of thymine bases. 11 types (HPLC grade purity) with different chain lengths (The "x" in dT(x) indicates the number of bases.) (dT(6), (10), (15), (16), (17), (18), (19), (20), (25), (30), and (40))
Concentration and Quantity	5 µmol/mL
Pretreatment	Dissolve the sample in water to prepare the concentrations indicated above.

Step 1: Initial Mobile Phase Screening

Sample	Mixture solution of 7 sequences (dT(6), (10), (15), (20), (25), (30), and (40))
Measurement Conditions	Ion-pair reagents were prepared using an aqueous acetic acid solution to adjust the pH of triethylamine (TEA), dibutylamine (DBA), and hexylamine (HA). Acetonitrile and methanol were used as organic solvents. 18 combinations of ion-pair reagents with different concentrations and organic solvents were evaluated using the analysis conditions listed in Table 1.
Results	Using TEA as the ion-pair reagent resulted in multiple coeluted peaks, regardless of the organic solvent combination (Figure 1). However, using DBA or HA improved separation. Both DBA and HA showed a tendency for separation to improve as the concentration was increased, but particularly HA showed a tendency to become more difficult to dissolve during preparation the higher its concentration.

Table 1 Analysis Conditions

System :	Nexera XS inert
Column :	Shim-pack Scepter Claris C18-120 (100 mm × 2.1 mm I.D., 3 µm) *1
Mobile phase A :	① 20/50/100 mmol/L TEA acetic acid aqueous solution pH6.5 ② 5/20/50 mmol/L DBA acetic acid aqueous solution pH6.5 ③ 20/50/100 mmol/L HA acetic acid aqueous solution pH6.5
Mobile phase B :	Acetonitrile or Methanol
Flow rate :	1.0 mL/min
B Conc. :	① TEA : 10-50 %(0-8 min) → 100 %(8.01-11 min) → 10 %(11.01-15 min) ② DBA : 20-70 %(0-8 min) → 100 %(8.01-11 min) → 20 %(11.01-15 min) ③ HA : 20-80 %(0-8 min) → 100 %(8.01-11 min) → 20 %(11.01-15 min)
Column temp. :	40 °C
Flow rate :	0.35 mL/min
Injection volume :	5 µL
Detection :	UV 260 nm (SPD-M40, UHPLC inert cell)
Vial :	TORAST-H Glass Vial*2

*1 P/N: 227-31210-05, *2 P/N: 370-04300-01 (Shimadzu GLC Ltd.)

Separation Condition Optimization Process

1. Screening	Ion-pair reagent types and concentrations Organic solvent types
2. Evaluating Conditions	Column oven temperature and mobile phase pH levels evaluated
3. Optimization	Gradient conditions

Parameters Considered

Alkylamine (type and concentration)

Methanol/acetonitrile mixture ratio

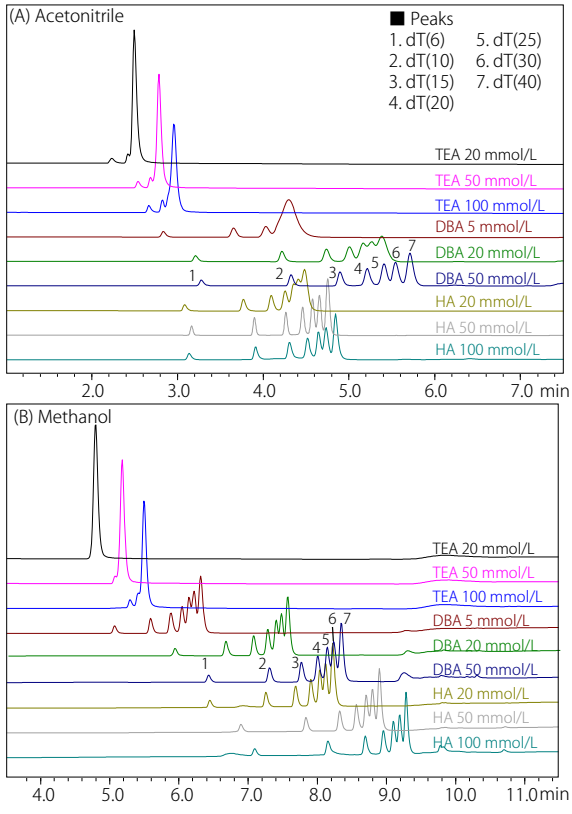


Figure 1 Chromatogram from Mixture Sample of 7 Oligonucleotide Components
Mobile Phase B: (A) Acetonitrile, (B) Methanol

Step 2: Optimization of Mobile Phase pH and Oven Temperature

Sample	Mixture solution of 7 sequences (dT(6), (10), (15), (20), (25), (30), and (40))
Measurement Conditions	Analysis conditions were further evaluated using 50 mmol/L DBA and 50 mmol/L HA as mobile phase A and using acetonitrile and methanol as the organic solvent. The gradient program was fixed as condition (2) in Table 1, whereas the column oven temperature was varied to 40, 50, 60, 70, and 80 °C and the mobile phase A pH level was varied to 6.0, 6.5, 7.0, and 7.5. The analysis batch was created and analyzed to comprehensively evaluate each combination. Since the retention time for each component varied significantly depending on parameter changes or organic solvent differences, the peak tracking functionality in LabSolutions MD was used to identify respective peaks. Due to the small differences between UV spectra for nucleic acid sequences, the information was not suited for identification. Instead, the elution order was used for identification.
Results	A design space was created with the pH of mobile phase A on the vertical axis and column oven temperature on the horizontal axis in order to visualize areas (Figure 2) where separation is at least 1.5 (or at least 1.2 only for combinations of HA and methanol). The black dots indicate measurement points, red areas indicate areas with high separation, and blue areas indicate low separation. In terms of the mobile phase pH level, the results suggest that different conditions are optimal depending on the organic solvent. In terms of column oven temperature, the results indicate that separation tends to increase the higher the temperature, regardless of the ion-pair reagent and organic solvent combination.

Step 3: Automatic Optimization of Gradient Conditions

Sample	Sample containing a mixture of 11 oligonucleotide sequences with different chain lengths (dT(6), (10), (15) to (20), (25), (30), and (40))
Measurement Conditions	Results were automatically searched to determine optimal gradient conditions that result in a minimum separation of 1.5. The organic solvent setting was fixed at methanol and the column oven temperature fixed at 80 °C. An AI algorithm was used to repeatedly perform corrected analyses based on two condition settings for mobile phase A (50 mmol/L DBA at pH 7.5 and 50 mmol/L HA at pH 7.5) in order to automatically search for gradient conditions that result in a minimum separation of 1.5 for respective mobile phase conditions. The resulting chromatograms after optimization are shown in Figure 4.
Results	Eventually, the respective mobile phase conditions were used to automatically search for gradient conditions that result in a minimum separation of 1.5. The resulting chromatograms after optimization are shown in Figure 4.

Functionality for Using AI to Automatically Optimize Gradient Conditions

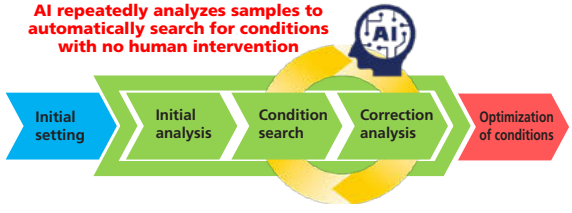


Figure 3 Automated Work ow of Gradient Optimization with LabSolutions MD

Figure 3 shows the LabSolutions MD process flow for automatically optimizing gradient conditions. LabSolutions MD includes a proprietary AI algorithm that can repeatedly make improvements to gradient conditions (condition search) and then analyze samples based on those corrected conditions (corrected analysis) to automatically search for conditions that satisfy separation requirements.

Parameters Considered

Mobile phase pH | Column oven temperature

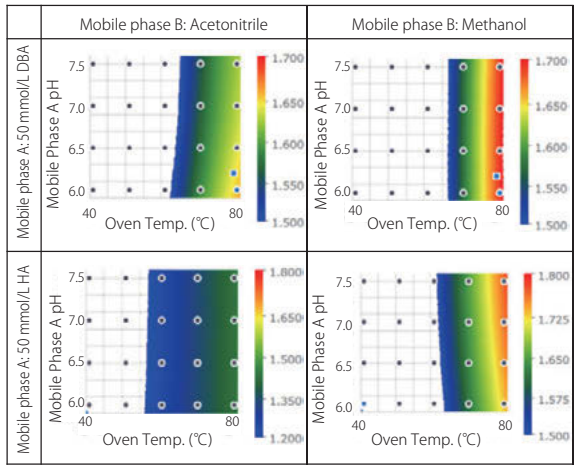


Figure 2 Design Space for Minimum Separation of 7 Oligonucleotide Components in Mixture Sample

Parameters Considered

Gradient conditions

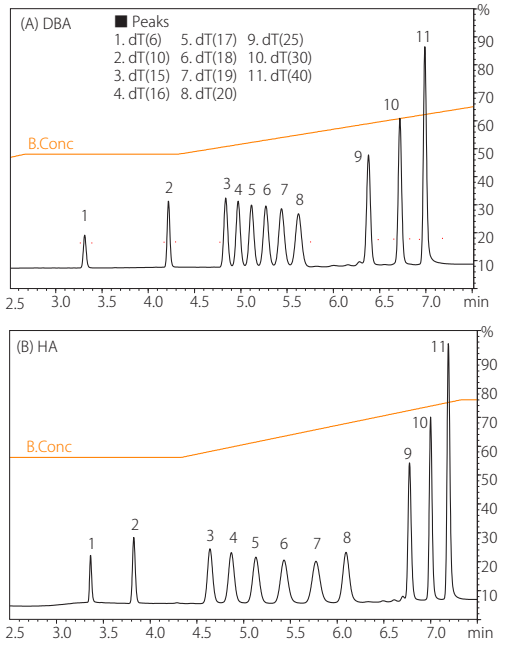


Figure 4 Chromatograms from Mixture Sample of 11 Oligonucleotide Components Using Automatically Searched Conditions
Mobile Phase A: (A) 50 mmol/L DBA pH 7.5
(B) 50 mmol/L HA pH 7.5

Conclusion

A standard mixture sample of 11 sequences with different chain lengths was used as a model sample for evaluating separation conditions for reverse-phase ion pair chromatography.

Using LabSolutions MD enabled a comprehensive evaluation of ion-pair reagent, mobile phase pH, and column oven temperature settings. Furthermore, visually showing the results was helpful for efficiently identifying optimal conditions. Furthermore, using the LabSolutions MD functionality for automatically optimizing gradient conditions, gradient conditions that satisfied the minimum 1.5 separation criterion were found within a few hours, which significantly reduced the amount of labor normally required for that process.

Efficient Method Development of Oligonucleotides by Reversed-Phase Ion-Pair Chromatography

Application

benefits

- LabSolutions MD can improve the efficiency of method development for oligonucleotides and related impurities.
- LCMS-2050 single quadrupole mass spectrometer can accurately track each peak of oligonucleotides and related impurities.
- Nexera XS inert (UHPLC system) with Shim-pack Scepter Claris (inert-coating metal-free column) offers complete inertness of the sample flow path to achieve optimal chromatographic separation of oligonucleotides.

Step 1: Initial Screening for Analysis Method

Table 1 Sequences of Oligonucleotide and Related Impurities

Name	Sequence (5'→3')	Length
FLP	T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*	20 mer
n-1(3')	T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*	19 mer
n-1(5')	mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*	19 mer
n-3	T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*	17 mer
n+1	T*-T*-mC*-T*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*	21 mer
PO	FLP (modified from phosphorothioate to phosphate diester at 5')	20 mer

* = 2'-O-methoxyethyl m = 5-methyl d = 2'-deoxy, PS(full)

Table 2 Analytical Conditions for Initial Screening

System :	Nexera XS inert (Method Scouting System)
Column :	Shim-pack Scepter Claris (100 mm × 2.1 mm I.D., 3 µm) *1
Temperature :	60 °C
Injection volume :	2 µL
Mobile phases Pump A – Line A :	100 mmol/L HFIP*2 and 20 mmol/L TEA*3 in water
– Line B :	100 mmol/L HFIP in water
– Line C :	200 mmol/L HFIP and 20 mmol/L TEA in water
– Line D :	200 mmol/L HFIP in water
Pump B – Line A :	Acetonitrile
– Line B :	Methanol
Flow rate :	0.4 mL/min
Time program (%B) :	6 % (0 min) →24 % (36 min)→ 50 % (36-37 min) →6 % (37-46 min)
Detection :	260 nm (SPD-M40, UHPLC inert cell)
System :	LCMS-2050
Ionization :	ESI/APCI (DUIS), negative mode
Mode :	SCAN (*m/z* 500-2000)
Nebulizing gas :	2.0 L/min (N_2)
Drying gas :	5.0 L/min (N_2)
Heating gas :	7.0 L/min (N_2)
DL temp. :	200 °C
Desolvation temp. :	450 °C
Interface Voltage :	-2.0 kV

*1 P/N : 227-31210-05 (Shimadzu GLC product number)
*2 1,1,1,3,3,3-hexafluoro-2-propanol
*3 Triethylamine

Parameters Considered

- Alkylamine concentration
- HFIP concentration
- Methanol/acetonitrile mixture ratio

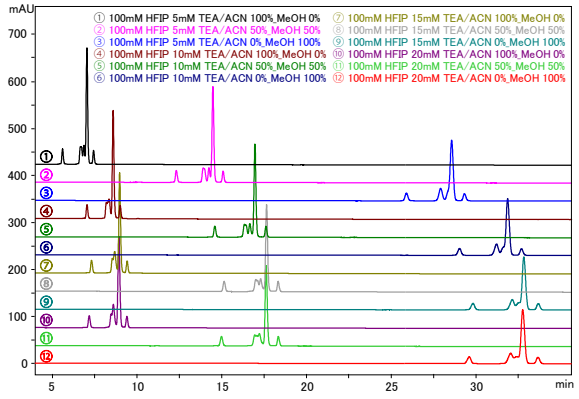


Figure 1 Chromatograms Obtained by Mobile Phase Screening 100 mmol/L HFIP (Upper)

Step 2: Optimization of Analysis Method

Parameters Considered

- Initial gradient concentration
- Acetonitrile concentration in organic mobile phase
- Column oven temperature

Automated Peak Tracking by LCMS-2050

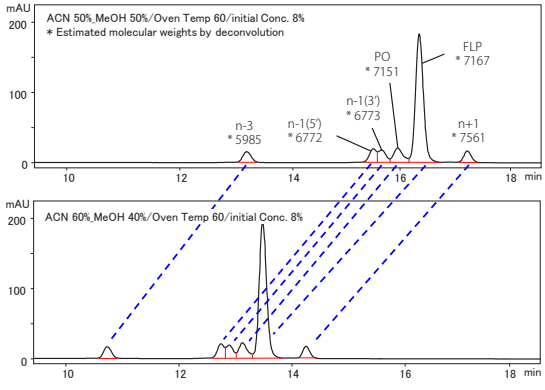


Figure 2 LC Chromatograms Obtained with 60 °C Column Oven Temperature, 8 % Initial Concentration, and 50 % (upper) or 60 % (lower) Acetonitrile Ratio (Dashed lines indicate impurity tracking based on molecular weight)

Design Space Evaluation for Optimal Condition

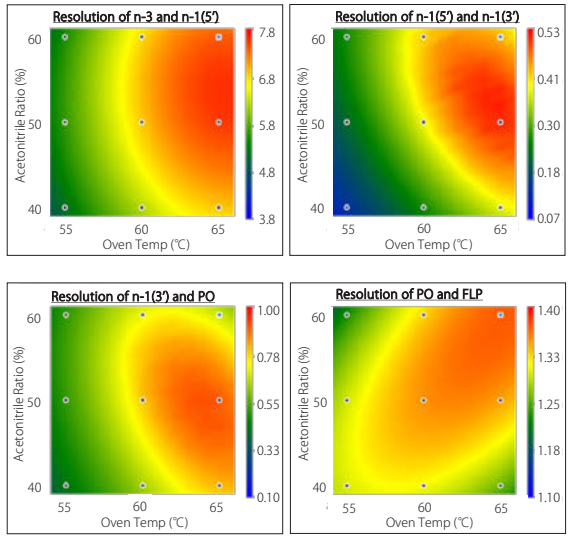


Figure 3 Design Space for Resolution of FLP and Related Impurities (Initial concentration is 8 %.)

Chromatogram at Optimal Condition

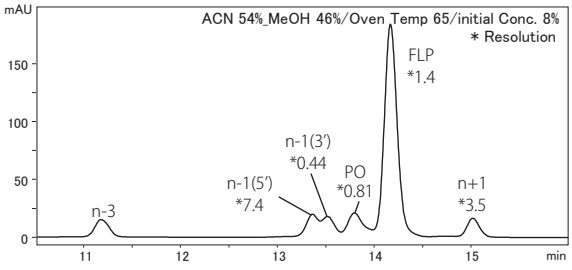


Figure 4 Chromatogram at Optimal Condition (100 mmol/L HFIP and 10 mmol/L TEA)

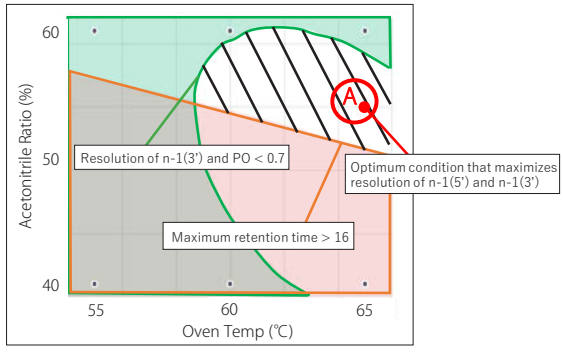


Figure 5 Overlay of Design Spaces of Resolution and Retention Time of Last Eluting Peak

Conclusion

The separation pattern of oligonucleotides is different depending on the concentration of HFIP and TEA in the aqueous mobile phase, the ratio of acetonitrile and methanol in the organic solvent, column oven temperature, and initial concentration of gradient program. The separation behavior can differ based on the structure of the oligonucleotide such as length, nucleobase, and the presence of modifications. Therefore, it is required to optimize the separation for each sequence of the oligonucleotide individually. On the other hand, a number of analyses and data processing for the optimization of analytical conditions is a time-consuming challenge. LabSolutions MD can automate the entire workflow, including the generation of an analysis schedule, the mobile phases preparation, and the data processing thanks to specific functionalities such as automated peak tracking, ranking of chromatograms by Evaluation Value, and design space. As this article describes, the combination of LabSolutions MD, Nexera XS inert, Shim-pack Scepter Claris, and LCMS-2050 strongly improves the efficiency of the overall workflow of method development for oligonucleotides.

Determining the Sequence of Drug Substances and Impurities

An Oligonucleotide Impurity Analysis Workflow Using LabSolutions Insight Biologics Software

Application

benefits

- The LCMS-9050 can be used with LabSolutions Insight Biologics data analysis software for sequencing oligonucleotides and impurities.
- All impurities can be comprehensively identified, including oligonucleotide user-specified modifications.
- The fragment coverage window in LabSolutions Insight Biologics makes it easy to determine the locations of deletions or modifications.

Methods and Results

Sample	20-mer oligonucleotide crude product with phosphorothioate modification Sequence: CTG CTA GCC TCT GGA TTT GA Monoisotopic mass: 6399.5998
Analysis	The sample was analyzed based on the analysis conditions listed in Table 1.
Analysis Software	LabSolutions Insight Biologics
Results	A comprehensive identification of all impurities in the MS1 data obtained resulted in finding over 30 impurities, such as chain length differences, base deletion variants, and ion adducts (Figure 1). Multi-charged ion analysis results for the PS20-mer peak are shown in Figure 2. The spectra of impurities were also similarly analyzed by multi-charged ion analysis in order to search for the impurities. Sequence coverage was checked for principal components and impurities with a deletion at 14 nucleotides from the 5′ terminal (present in 0.5 % of principal components) based on fragment spectra obtained from MS2 data. This confirmed a high coverage rate (Figure 3).

Table 1 Analysis Conditions

[HPLC conditions] (Nexera XS inert)	
Column :	Shim-pack Scepter Claris C18-120, 100 mm x 2.1 mm I.D., 1.9 µm
Mobile phase A :	Aqueous solution of 100 mM HFIP and 10 mM TEA
Mobile phase B :	50 % Methanol solution of 50 mM HFIP and 5 mM TEA
Gradient program :	5 %B (0-1 min)→ 40 %B (26 min) → 90 %B (26.1-30 min) → 5 %B (30.1-34 min)
Column temp. :	60 °C
Injection volume :	2 µL
[MS conditions] (LCMS-9050)	
Ionization :	ESI (Negative mode)
Mode :	MS scan (*m/z* 550~2500), DDA
Interface voltage :	-3.0 kV
Nebulizing gas flow :	3.0 L/min
Drying gas flow :	10.0 L/min
Heating gas flow :	10.0 L/min
DL temp. :	250 °C
Block heater temp. :	400 °C
Interface temp. :	350 °C

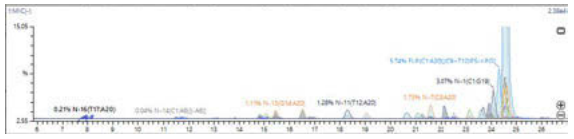


Figure 1 Component Chromatogram

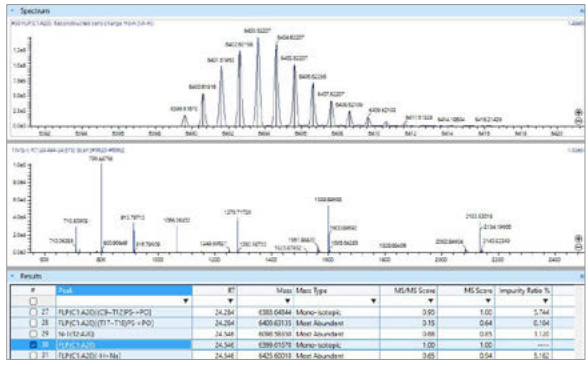


Figure 2 Multi-Charged Ion Analysis Results
Upper: Multi-Charged Ion Analysis Spectrum; Middle: Mass Spectrum; Lower: Identification Results

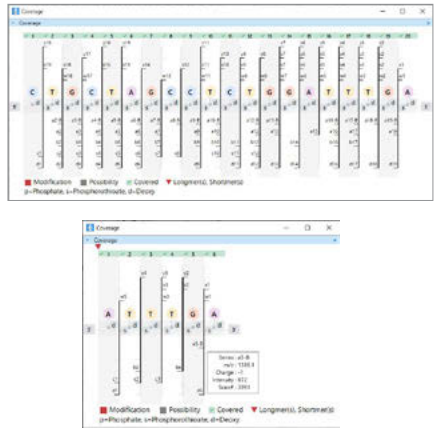


Figure 3 Sequence Coverage
Upper: Principal Components; Lower: Impurities with Deletion at 14 Nucleotides from the 5′ Terminal

Conclusion

LabSolutions Insight Biologics software can comprehensively characterize and identify the sequences of oligonucleotide impurities.
The analysis workflow described in this article achieved complete sequence coverage not only for the main sample component, but also for an impurity with a relative abundance of 0.5 % compared with the main component.

Quadrupole Time-of-Flight Liquid Chromatograph Mass Spectrometer

LCMS-9050

Product

Features

The LCMS-9050 is a QTOF mass spectrometer designed for reliable and easy acquisition of accurate masses in real laboratory settings.

Trusted mass accuracy

Shimadzu's proprietary high-accuracy temperature control system suppresses even tiny mass variations caused by external factors, ensuring accurate mass measurements without the operator worrying about mass calibration.

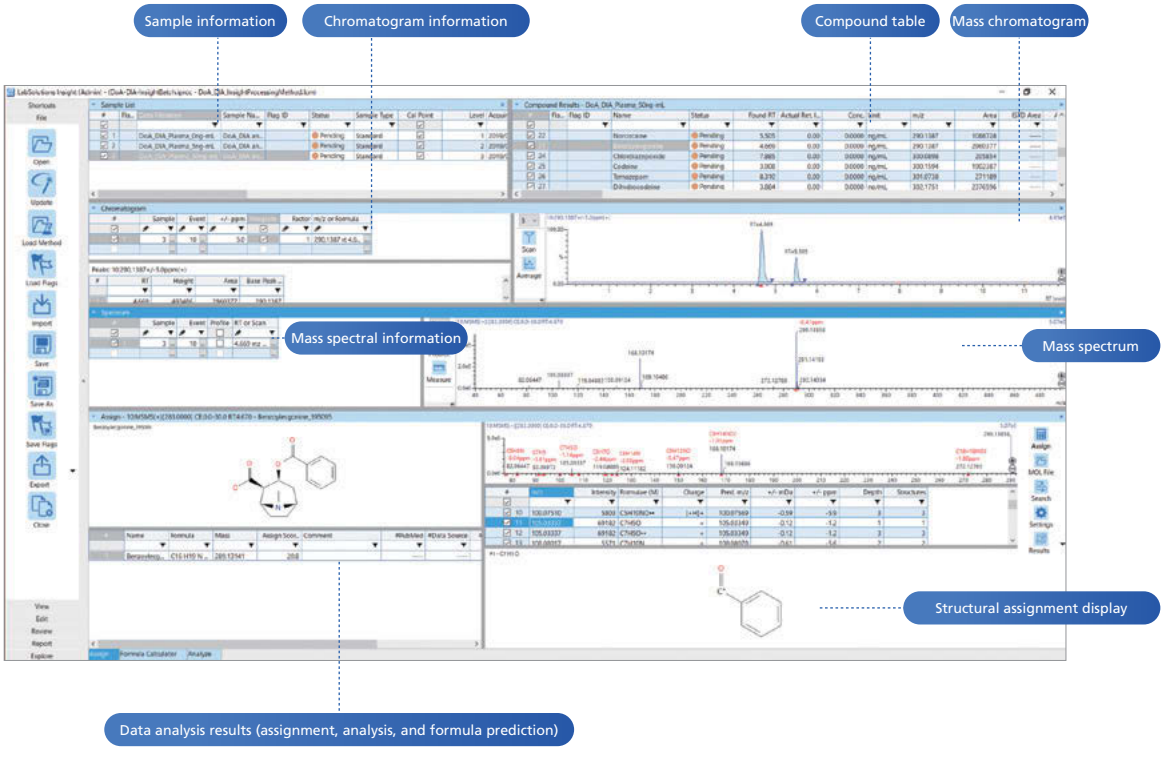
Ultra-stable polarity switching

Reliable high-speed positive-negative polarity switching technology enables simultaneous analysis of both positive and negative ions and contributes to the development of new applications and more efficient analysis.

LabSolutions Insight Explore

Software for Supporting Everything from Qualitative to Quantitative Analysis

Explore software not only facilitates quantitative analysis of a large number of samples, but also comes with features for reliable library searching, elucidation of compound structure and sample composition, and multivalent ion analysis, making full use of MS data with high resolution and mass precision.



Multivalent Ion Analysis (Deconvolution)

For the deconvolution of multiple charge state envelopes produced by large molecules, such as proteins and oligonucleotides, LabSolutions Insight Explore CSD (option) includes "ReSpect". This multivalent ion analysis algorithm from Positive Probability Ltd detects group(s) of multi-charged peaks and calculates the mass of the original species.

Reversed-Phase Ion-Pair LC/MS Analysis of siRNA under Denaturing and Non-Denaturing Conditions

Application

benefits

- The LCMS-9050 quadrupole time-of-flight mass spectrometer can be used to characterize siRNA.
- The elution of siRNA in double-stranded (non-denatured) or single-stranded (denatured) state can be confirmed by changing the column temperature.
- The LabSolutions Insight Biologics analysis software enables analysis of multiple oligonucleotide sequences at once.

Samples

Double-stranded siRNA annealed with sense and antisense strands of the following sequenceswas used.
Sense:
G-Um-A-A-Cm-Cm-A-A-G-A-G-Um-A-Um-Um-Cm-Cm-A-Um-dT-dT
Antisense:
A-U-G-G-A-A-Um-A-C-U-C-U-U-G-G-U-Um-A-C-dT-dT
(d: 2'-deoxy and m: 5-methyl)

Configuring the Data Analysis Parameters

LabSolutions Insight Biologics can analyze multiple sequences. As an example, in Figure 1, the sequences of sense and antisense strands were set as analysis targets. The specified sequences and optionally added information about nucleobases, linkers, riboses, and base modifications can be saved as an analysis settings file.

Analytical Conditions

Analysis was performed with Nexera XS inert UHPLC and LCMS-9050 quadrupole time-of-flight mass spectrometer systems. The analytical conditions are shown in Table 1.

Table 1 Analysis Conditions

UHPLC (Nexera XS inert)	
Column :	Shim-pack Scepter Claris C18-300*1 (100 mm x 2.1 mm I.D., 1.9 µm,)
Mobile Phase A :	100 mM HFIP, 10 mM TEA - water
Mobile Phase B :	100 mM HFIP, 10 mM TEA - methanol
Gradient Program :	B Conc. 5 % (0 min) - 50 % (10 min) - 90 % (10.01-12 min) - 5 % (12.1-25 min)
Flowrate :	0.3 mL/min
Column Temp. :	25 or 60 °C
Injection Volume :	1 µL

*1: P/N 227-31209-02

MS (LCMS-9050)	
Ionization :	ESI negative
Mode :	MS *m/z* 550-2500
Nebulizing Gas Flow :	2.0 L/min
Drying gas Flow :	10.0 L/min
Heating gas Flow :	10.0 L/min
Interface Temp. :	350 °C
DL Temp. :	250 °C
Block Heater Temp. :	400 °C

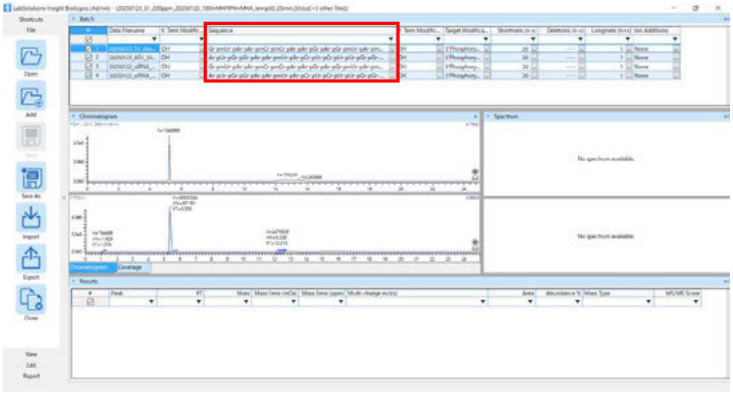


Figure 1 Example of Multiple Sequence Settings

Separation of siRNA under Denaturing and Non-Denaturing Conditions

The UV chromatograms of sense, antisense, and doublestranded siRNA are shown in Figure 2. The sense and antisense strands were eluted at different retention times at both 60 and 25 °C column oven temperatures. At a 60 °C column oven temperature, double-stranded siRNA was detected as denatured and dissociated into sense and antisense strands. On the other hand, at a 25 °C column temperature, doublestranded siRNA was eluted at a retention time of 7.5 minutes, which was slower than that of sense and antisense strands. In addition, the peak of the sense strand, which seemed to remain without forming a duplex during annealing, was also observed.

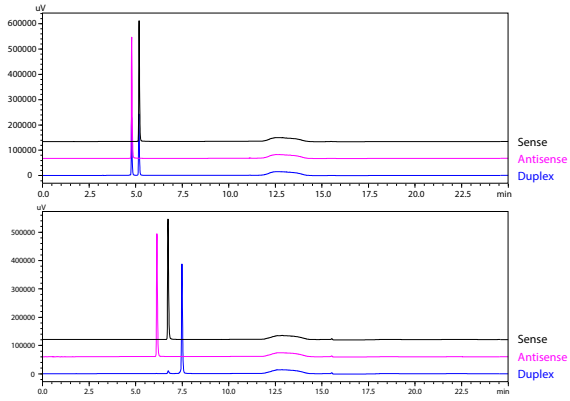


Figure 2 UV Chromatograms (260 nm) of the Sense Strand, Antisense Strand and Duplex siRNA
Top: With 60 °C Column Oven Temp.; Bottom: With 25 °C Column Oven Temp.

Results of Identification by LC/MS

Figure 3 shows the component chromatograms obtained by LC/MS analysis of double-stranded siRNA. In Insight Biologics, the identified oligonucleotide sequence is displayed as a component chromatogram based on the MS1 spectrum and summed with different valences and isotopes. The sense strand and antisense strand sequences were identified at both column temperatures of 60 °C and 25 °C.

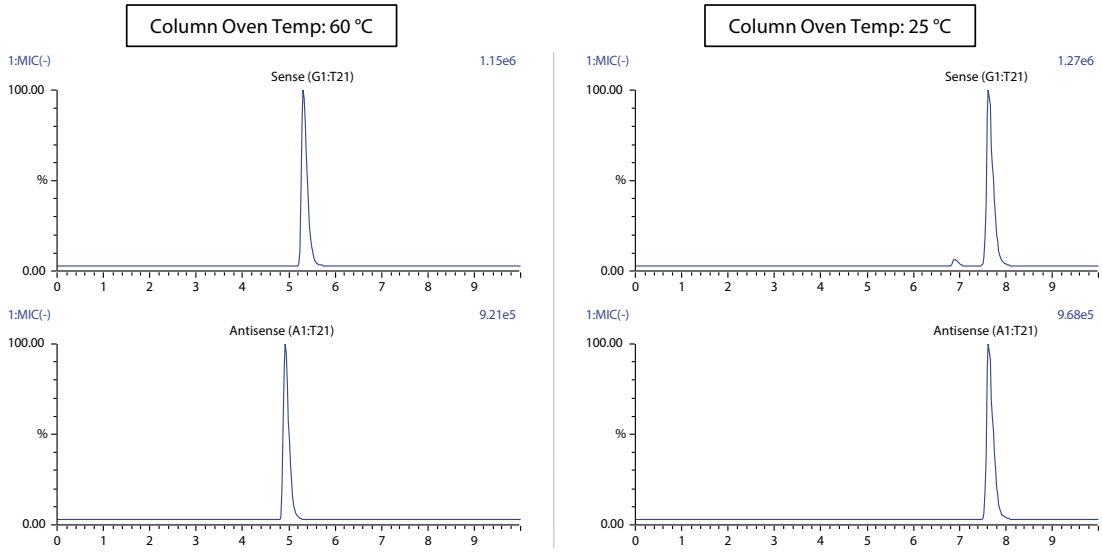


Figure 3 Component Chromatograms of Duplex siRNA (Results of Identification by LC/MS)
Left: With 60 °C Column Oven Temp.; Right: With 25 °C Column Oven Temp.

Conclusion

LC/MS analysis of siRNA under denaturing and non-denaturing elution conditions was performed using an LCMS-9050 quadrupole time-of-flight mass spectrometer. Under denaturing conditions at a column temperature of 60 °C, siRNA was eluted in a dissociated single-stranded state, but at a column temperature of 25 °C, siRNA was eluted in a double-stranded state. By using the oligonucleotide analysis software LabSolutions Insight Biologics, multiple nucleic acid sequences can be analyzed at the same time.

<Acknowledgments>
This research was supported by AMED under Grant Number JP21ae0121022, JP21ae0121023, JP21ae0121024 (Project leader: Satoshi Obika).

Sequence Analysis of Antisense Oligonucleotides Using a MALDI-8030 Benchtop MALDI-TOF Mass Spectrometer

Application

benefits

- Oligonucleotide therapeutics can be analyzed using a simple and affordable benchtop instrument.
- MALDI-ISD enables simple and reliable sequencing of antisense oligonucleotides.
- There is no need to interpret complicated MS/MS spectra, which saves labor and time.

Methods and Results

Sample	Synthesized oligonucleotides modeled after 3 types of antisense oligonucleotide therapeutics (mipomersen, nusinersen, and inotersen) (Table 1)
Concentration and Quantity	Each sample was dissolved in water to achieve a concentration of 0.1 mg/mL (about 13 to 14 pmol/µL).
Mass Spectrometry	0.5 µL of each sample solution was placed on a MALDI target plate, followed by layering 0.5 µL of matrix solution on top. The samples were air-dried and analyzed using a MALDI-8030 system. Matrix solutions consisted of either 40 mg/mL THAP (2',4',6'-tri hydroxyacetophenone monohydrate) or HPA (2-hydroxypicolinic acid), both dissolved in 50% acetonitrile with 40 mM ammonium citrate as an additive.
Results	Molecular Weight Measurement HPA or THAP is commonly used as a matrix. When they were also considered for the model oligonucleotide therapeutic in this case, they were successfully ionized in the negative ion mode as a deprotonated [M-H]$^-$ form. Adding ammonium citrate helped inhibit any unwanted cation adducts (Figure 1). Sequence Confirmation In MALDI-ISD analysis of oligonucleotides, a-series ions from the 5' side and w-series ions from the 3' side, both resulting from cleavage at a single phosphodiester bond, are uniquely observed (Figure 2). Using HPA as the matrix, it was found that oligonucleotide therapeutics with phosphorothioate and various other chemical modifications still generate a continuous series of ISD peaks (Figure 3). Due to the simplicity of the mass spectra composed of singly-charged ions, manual analysis is possible, but automated analysis can also be performed using LabSolutions Insight Biologics.

Molecular Weight Confirmation

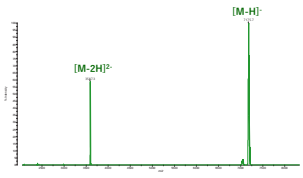


Figure 1 Negative-Ion MALDI Mass Spectrum of Mipomersen Using THAP as a Matrix

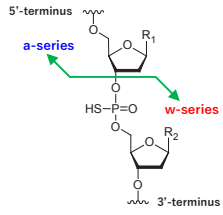


Figure 2 Ion Fragmentation Nomenclature of Oligonucleotides Observed from MALDI-ISD

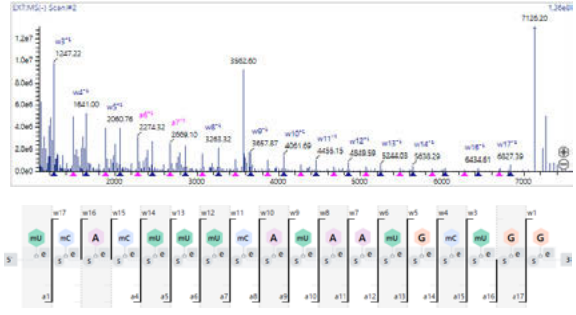


Figure 3 Example of the result of a model oligonucleotide therapeutic analysis using LabSolutions Insight Biologics.

Table 1 Three Types of Model Compounds

Name	Chemical formula	Mw	Note
Mipomersen	C230H324N67O122P19S19	7177.2	ASO, PS (full), 2'-MOE, 20 mer
5'-G*-mC*-mC*-mU*-mC*-dA-dG-dT-dmC-dT-dG-dmC-dT-dT-dmC-G*-mC*-A*-mC*-mC*-3'			
Nusinersen	C234H340N61O128P17S17	7127.2	ASO, PS (full), 2'-MOE, 18 mer
5'-T*-mC*-A*-mC*-T*-T*-T*-mC*-A*-T*-A*-A*-T*-G*-mC*-T*-G*-G*-3'			
Inotersen	C230H318N69O121P19S19	7183.1	ASO, PS (full), 2'-MOE, 20 mer
5'-T*-mC*-T*-T*-G*-dG-dT-dT-dA-dmC-dA-dT-dG-dA-dA-A*-T*-mC*-mC*-mC*-3'			

* = 2'-O-(2-methoxyethyl) m = 5-methyl d = 2'-deoxy

Conclusion

Because MALDI preferentially produces singly-charged ions, mass confirmation of synthetic oligonucleotides can be achieved easily without interpreting complicated mass spectra. Furthermore, the ability to perform MALDI-ISD measurements with an instrument lacking MS/MS capability means the internal sequence of oligonucleotides can be confirmed using a simple benchtop-type linear MALDI-TOF MS system. That also enables the sequencing of model oligonucleotide therapeutics that contain various chemical modifications based on the consecutive ISD fragment ions originating from a simple cleavage.

<Acknowledgements>
This research was supported by AMED under Grant Number JP21ae0121022, JP21ae0121023, JP21ae0121024 (Project leader: Satoshi Obika).

Dual-Polarity Benchtop Linear MALDI-TOF Mass Spectrometer

MALDI-8030/MALDI-8030 Easycare

Product

Features

The MALDI-8030 is a benchtop linear MALDI-TOF mass spectrometer with a compact footprint. It is used for quality control and profiling of peptides, proteins, polymers and oligonucleotides. It is equipped with an automated, rapid UV laser-based ion source cleaning function (TrueClean) that can clean the ion extraction electrode without breaking vacuum.

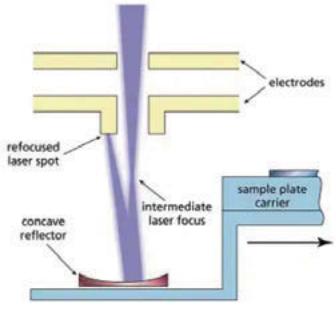
- Dual-polarity linear mode MALDI-TOF (positive- and negative-ion mode)
- 200 Hz solid-state laser, 355 nm
- Load-lock chamber for fast sample introduction
- UV laser-based source cleaning (Pat. US 10340131)
- Small footprint/benchtop design
- Quiet operation (<55 dB)

Sample Target Solutions

Compatible with the FlexiMass series of microscope slide-format sample targets, these slides provide researchers with options that align with their experimental workflow. The individually barcoded, single-use FlexiMass-DS slides provide a convenient solution for more routine or defined workflows. Ready-to-use, these disposable targets eliminate the need for cleaning and the risk of carryover. Alternatively, the reusable stainless steel FlexiMass-SR sample targets provide a cost-effective, longer-term solution to sample preparation.

Automated Source Cleaning TrueClean

To maintain instrument performance over time, the MALDI-8030 features wide-bore ion optics which minimize the risk of source contamination and provide a robust platform. TrueClean, an automated source cleaning function, cleans the extraction electrode in-situ quickly within 10 min by using a UV laser without breaking instrument vacuum.



High Quality with Low-running Costs

For improving product performance and longevity, components were carefully selected to realize a robust platform capable of delivering outstanding performance in a small footprint. The simple design provides easy maintenance and contributes to maintaining the quality of analysis over time.

Database Management

MALDI Solutions software features a centralized, secure Microsoft SQL database which can be used to store everything from sample lists and acquisition Parameter Sets to acquired MALDI data. The system is managed by an administrator and customizable user profiles provide control over access to the database and operation of the instrument.

Oligonucleotide Analysis Using the Compact MALDImini-1 MALDI Digital Ion Trap Mass Spectrometer

Application

benefits

- Using a MALDImini-1 spectrometer in combination with LabSolutions Insight Biologics software, both molecular weight and sequences can be analyzed, with process steps from measurement to data analysis completed within about 10 minutes.
- The compact MALDImini-1 system enables space-efficient operation within facilities with limited space.

Methods and Results

Sample	Synthetic oligonucleotide used as a model sequence for an antisense oligonucleotide (nusinersen) (For the sequence, refer to the MALDI-8030 information on p. 22.)
Concentration and Quantity	A 20 pmol/µL aqueous solution was prepared as the sample solution.
Analysis	After preparing a 1:1 mixture (v/v) of sample and matrix solutions, 1 µL of the mixture was spotted onto a sample plate, dried, and measured. Matrix solutions were prepared by dissolving 40 mg/mL concentrations of 3-hydroxypicolinic acid (3-HPA) and 2,4-dihydroxyacetophenone (2,4-DHAP) respectively in a 50/50 acetonitrile/water (v/v) solution that contains 70 mM diammonium hydrogen citrate. A 3-HPA/2,4-DHAP mixture matrix solution was prepared by mixing a 1:1 (v/v) ratio of the 3-HPA and 2,4-DHAP solutions. Samples were measured by raster scans using the MALDImini-1. Separate condition settings were used for molecular weight analysis and sequence analysis.
Results	• The molecular weight analysis detected $[M+H]^+$. • The sequence analysis detected a large number of mostly singly-charged fragment ions limited to the series of fragment ions cleaved from a specific linkage. • Data acquired by MALDImini-1 molecular weight analysis and sequence analysis was output and analyzed using LabSolutions Insight Biologics software to quickly obtain identification results. The predicted sequence displayed based on MALDImini-1 fragment characteristics was simple.

Molecular Weight Analysis Results

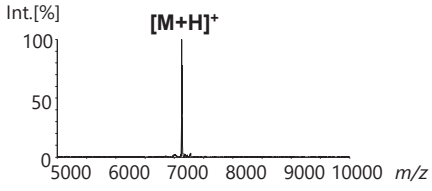


Figure 1 Mass Spectrum Measured by MALDImini-1

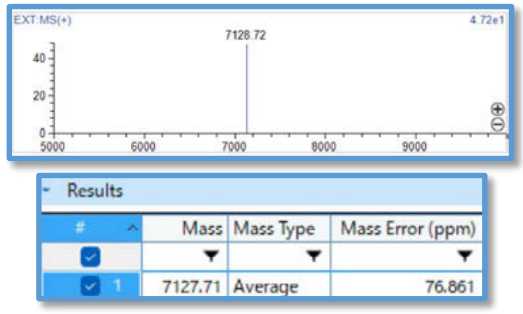


Figure 2 Part of Results from Using LabSolutions Insight Biologics to Analyze MALDImini-1 Data

Sequencing Results

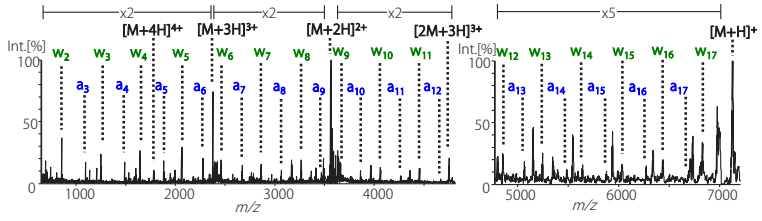


Figure 3 Fragment Spectra using MALDImini-1

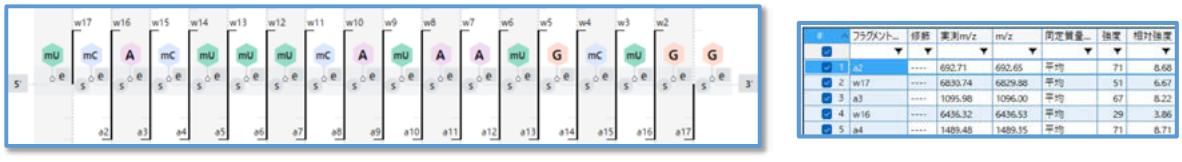


Figure 4 Example of Partial MALDImini-1 and LabSolutions Insight Biologics Data Analysis Results, Sequence Coverage (left) and Identification Results (right) Window Display, where s = Thiophosphorate, e = Methoxyethoxy, and m = Methyl

Conclusion

Analysis using the compact MALDImini-1 spectrometer with LabSolutions Insight Biologics oligonucleotide sequencing software enables highly reliable sequence analysis based only on singly-charged ions. The space-efficient system provides quick and easy sequencing analysis.

MALDI Digital Ion Trap Mass Spectrometer

MALDImini-1

Product

Features

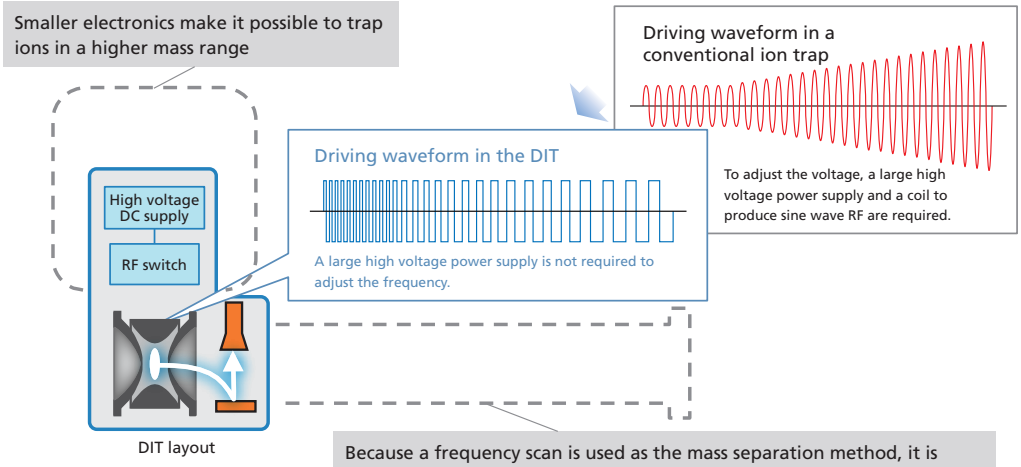
Despite its light and compact shape, the MALDImini-1 allows high-sensitivity MS^n measurements, making it suitable for a large number of applications.

The MALDI-MS that can be installed anywhere

Until now, devices for MS^n have had strenuous installation requirements, but the MALDImini-1's compact size and power requirements make it easy to install almost anywhere.
Easy access to the instrument allows users to quickly acquire data and check their results, creating a more convenient workflow right on the bench.

Digital Ion Trap (DIT) technology provides high sensitivity over a wide mass range, despite the device's small footprint

The world-first* Digital Ion Trap (DIT) technology, unique to Shimadzu, uses rectangular wave RF to trap ions rather than the sine wave RF which has been used in ion-trapping devices until now.



＊As of May 2019, from internal research

Begin taking measurements quickly

MALDI lends itself to quick analysis through simple sample preparation and acquisition. Once the sample is loaded, users can acquire a spectrum in a few minutes at the click of a button.
With such a smooth and quick setup, it is possible to carry out detailed structural analyses in a very short time span.

Place the sample onto the plate
(FlexiMass™-SR or FlexiMass-DS can be used)

Insert the plate into the device and wait for the vacuum

Begin analysis

MS^n analysis is possible over a wide mass range, even with microquantity samples

By combining a MALDI ion source with Digital Ion Trap (DIT) technology, it is possible to carry out high-sensitivity MS and MS^n analysis even on micro-quantity samples. Not only can the mass of various molecules be checked, but it is also possible to carry out a wide range of analyses such as identifying proteins and investigating the structure of glycans and glycopeptides.

Thermal Stability Analysis of Oligonucleotides by New Tm Analysis System

Application

benefits

- Easily determine the temperature at which 50 % of double-stranded nucleic acids dissociate into single strands (Tm value).
- Automate annealing (pretreatment) and Tm value analysis using the average and derivative methods.
- Achieve the industry's highest data integrity when linked to the LabSolutions DB/CS system.

Methods and Results

Sample Forward Reverse	M13 primer 5'-CGACGTTGTAAAACGACGGCCAGGA-3' 5'-TCCTGGCCGTCGTTTTACAACGTCG-3'
Concentration	12 µM of 10 mM phosphate buffer (containing 17 mM NaCl)
Analysis Conditions	Listed in Table 1
Results	Results calculated by two methods (median method and differential method) are shown in Table 2. The median method calculates the Tm value (melting temperature) as the point where the absorbance curve intersects the median line between two lines tangent to respective segments selected in the regions before and after the transition region. The Tm value was determined by arbitrarily specifying the regions before and after the transition, as shown in Figure 1. The differential method calculates the first-order derivative of each point with the specified interval and determines the Tm value (melting temperature) as the point where the maximum value occurs. The evaluated region was specified arbitrarily to determine the Tm value, as shown in Table 2.

Table 1 Measurement Conditions

Instruments:	UV-2600i、TMSPC-8
Measuring Wavelength:	260 nm
Measuring Wavelength (for Calibration):	320 nm
Slit Width:	2.0 nm
Temperature Range:	15 to 90 °C
Acquisition Rate:	1 °C
Temperature Changing Speed:	1 °C / min
Cell:	8-cell micro cell with 1 mm optical path length

Table 2 Measurement Results of the Sample

Analysis Method	Tm value (°C)
Average Method	65.47
Derivative Method	65.98

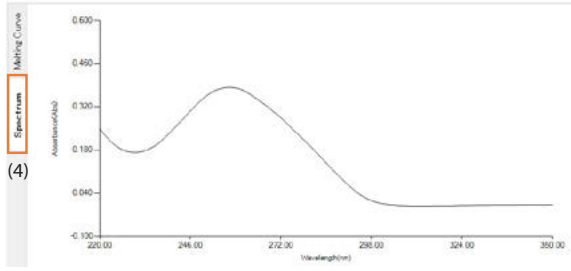


Figure 1 Absorption Spectrum

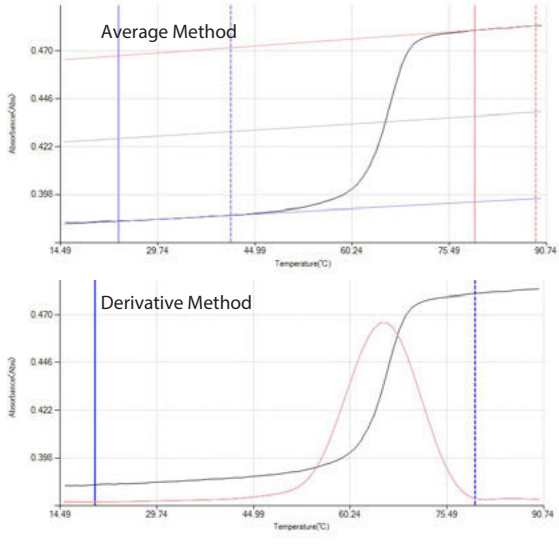


Figure 2 Absorption Spectra of Sample (Upper: Median Method; Lower: Differential Method)

Conclusion

The Tm analysis software can be used to calculate Tm values for evaluating the quality or stability of double-stranded DNA or RNA. In addition, calculated Tm values for a variety of concentrations (C) can be used for more detailed hybridization analysis.

UV-Vis Spectrophotometer

Tm Analysis System

Product

Features

Tm analysis system, TMSPC-8, is connected to a spectrophotometer and measures the Tm of oligonucleotides by heating and cooling. The rate of temperature increasing/decreasing can be selected from 12 levels of ± 0.1, 0.2, 0.5, 1, 2 and 5 °C. There are 2 types of 8 micro-multi-cells with optical path lengths of 10 mm (minimum sample volume of 100 µL) and 1 mm (minimum sample volume of 10 µL), and these cells allow measuring 8 types of samples simultaneously in parallel.

Reliable Data Integrity

The Tm analysis system in conjunction with LabSolutions DB/CS can achieve the highest data integrity levels in the industry. Measurement parameters, audit trails, and results of data acquisition and analysis are managed in a database protected with user privilege settings and security policies to prevent unintended operations or data tampering by operators.

Functionality for Satisfying Various Needs

In addition to trace measurement and high-sensitivity measurement capabilities required for Tm analysis, the system satisfies a variety of other needs, including thermodynamic parameter analysis using functionality for automatically transferring data to an Excel file.

Efficient Automated Workflow

Conventional Tm analysis requires multiple steps, including checking the UV-Vis spectrum, annealing, measuring the melting curve, and analyzing data, with recording and data management performed separately.
In contrast, Tm Analysis System achieves a seamless workflow and efficient Tm analysis by performing time-consuming annealing, correction (background wavelength and temperature blank correction), and Tm value calculation (average or derivative methods) steps automatically.

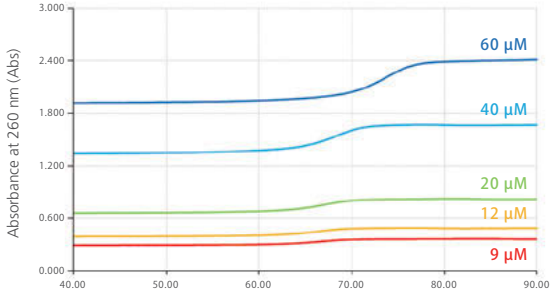
8-Cell Micro Multi-Cell

This micro multi-cell can be used to measure eight samples at the same time using the same measurement conditions.
In addition to previous micro multi-cell models with a 10 mm optical path length (with a 100 µL minimum sample volume) for satisfying high-sensitivity measurement needs, a new model with a 1 mm optical path length (with a 10 µL minimum sample volume) for measuring micro-quantities of expensive samples has been added to the lineup. Cells with a 1 mm optical path length can result in sample evaporation problems, but a new sealing method*, which suppresses the evaporation of samples by sealing the top side of the cell, enables reliable measurement of samples with high melting temperatures.

*The sealing method was supported by Professor Junji Kawakami (Department of Nanobiochemistry, Faculty of Frontiers of Innovative Research in Science and Technology (FIRST), Konan University, Japan). This was supported by AMED under Grant Number JP21ae0121022, JP21ae0121023, JP21ae0121024 (Project leader: Satoshi Obika).

1 mm Optical Path Length
Micro Multi-Cell

10 mm Optical Path Length
Micro Multi-Cell



Melting Curve of Nucleic Acid (M13 Primer)
(Using Micro Multi-Cell with 1 mm Optical Path Length)

Efficient Analysis of Residual Solvents in Pharmaceuticals Using the Compact Model, Brevis GC-2050 —JP18 and USP467, Water-Soluble Samples—

Application

Note: The data shown is not from analyzing an oligonucleotide therapeutic.

benefits

- The slim and compact design of the Brevis GC-2050 enables the expansion of the number of operational units in the laboratory, allowing for efficient high-throughput analysis.
- Brevis GC-2050 can perform analysis using alternative carrier gases.
- Analysis can be performed with tert-butyl alcohol and cyclopentylmethyl ether, which have been newly added as Class 2 solvents in ICH Q3C (R8).

Methods and Results

Sample	Class 1 and 2 standard solutions (water-soluble samples)
Analysis Conditions	Listed in Table 1.
Results	Results from analyzing a class 1 standard solution (water-soluble sample) by operating method A are shown in Figure 1 and results from analyzing a class 2 standard solution (water-soluble sample) are shown in Figure 2 (with class 2A: black; class 2B: pink; TBA, CPME, and MiBK: blue). For class 1 residual solvents, good S/N ratios were obtained in carbon tetrachloride analysis required for checking the sensitivity. For class 2 residual solvents, good system suitability results were obtained both operating method A (1.0 or greater separation between acetonitrile and dichloromethane peaks) and operating method B (1.0 or greater separation between acetonitrile and cis-1,2-dichloroethene peaks).

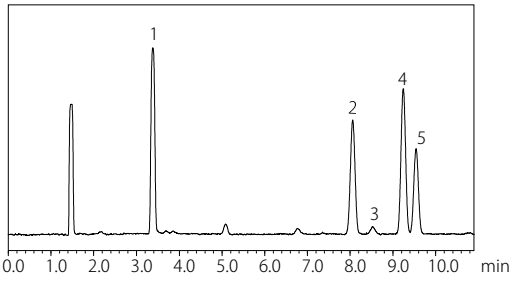


Figure 1 Class 1 Standard Solution Chromatogram (Water-Soluble Sample) Using Procedure A.
1,1-Dichloroethane 2. 1,1,1-Trichloroethane
3.Carbon tetrachloride 4.Benzene 5.1,2-Dichloroethane

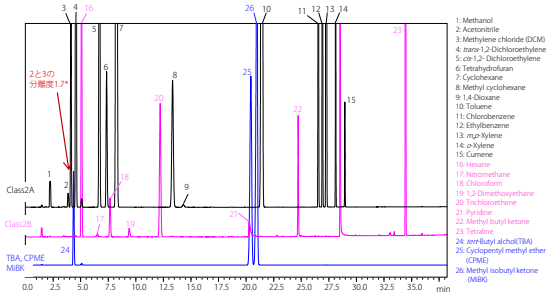


Figure 2 Class 2 Standard Solution Chromatogram (Water-Soluble Sample) Using Procedure A
Note: Separation values shown are intended for reference purposes and are not guaranteed.

Table 1 Analytical Conditions

GC Analytical Conditions (Procedure A and B)	
Model :	Brevis GC-2050
Detector :	FID (Flame Ionization Detector)
Column :	A) SH-I-624Sil MS (0.32 mm I.D.×30 m, d.f.= 1.8 µm) B) SH-PolarWax (0.32 mm I.D.×30 m, d.f.= 0.25 µm)
Column Temperature :	A) 40 °C (20 min) –10 °C/min –240 °C (20 min) Total 60 min B) 50 °C (20 min) –6 °C/min –165 °C (20 min)Total 59.17 min
Injection Mode :	A) Split 1:5 B) Split 1:10
Carrier Gas Controller :	Linear velocity (He, N_2, H_2)
Linear Velocity :	35 cm/sec
Detector Temperature :	250 °C
FID H_2 Flow Rate :	32 mL/min
FID Make up Flow Rate :	24 mL/min (N_2)
FID Air Flow Rate :	200 mL/min
Injection Volume :	1 mL
HS-20 NX Analytical Conditions (Same for Procedure A and B)	
Model :	HS-20 NXUSTL (Ultra Short Transfer Line)
Oven Temperature :	80 °C
Sample Line Temperature :	110 °C
Transfer Line Temperature :	120 °C
Vial Shaking Level :	Off
Vial Volume :	20 mL
Vial Equilibrating Time :	60 min
Vial Pressurizing Time :	1 min
Vial Pressure :	75 kPa
Loading Time :	0.5 min
Load Equilib. Time :	0 min
Needle Flush Time :	5 min

Conclusion

Even though the Brevis GC-2050 is small and compact, it is capable of analyzing residual solvents in pharmaceuticals in accordance with the JP18 and USP General Chapters<467> Residual Solvents. That compactness means the number of units installed in a laboratory can be increased compared with high-end models, so residual solvents in pharmaceuticals can be efficiently analyzed.

Gas Chromatograph

Brevis GC-2050

Product

Features

Despite its compact size, the Brevis GC-2050 achieves uncompromised analytical performance that satisfies market needs for "a smaller, simpler, and easier to use model that can be reliably operated in a laboratory without sacrificing analytical performance."

Brevis GC-2050, HS-20 NX USTL (Ultra Short Transfer Line)

Compact without Compromises

The compact size with a width of only 350 mm increases the number of units that can be operated in confined spaces while still providing the expandability necessary for satisfying a wide variety of analytical needs. Despite its space-efficient design, it can hold two typical capillary columns and also supports using hydrogen or other helium alternatives as the carrier gas.

Built-in Analytical Intelligence

Using Remote Display and LabSolutions Direct functionality, GC systems can be accessed from anywhere using a smart device (automatic remote operations). Clean Pilot functionality can be used to automatically condition columns quickly and efficiently to ensure consistent analytical results while reducing laboratory workloads.

Best-in-Class Performance

It achieves extremely high area and retention time reproducibility while maintaining the same performance levels as top-of-the-line models.

Headspace Sampler

HS-20 NX

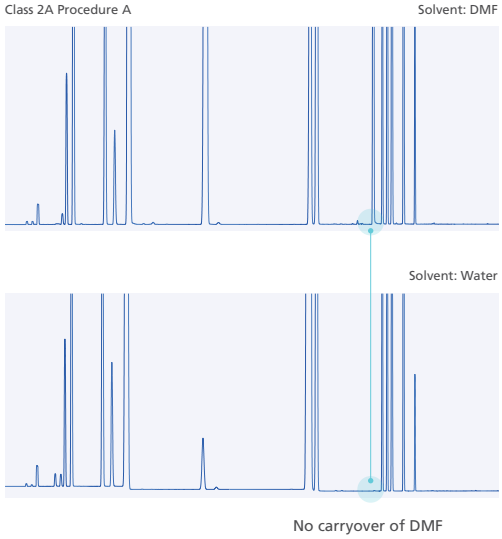
Product

Features

As an ideal solution for analyzing volatile components, the high basic performance level and user-friendly design offer powerful backup support for a wide variety of analytical applications in organizations ranging from research departments to quality control.

Ultra-low carryover

The HS-20 NX series uses the isolation gas flow to reduce carryover to 1/10 of conventional models. It supports a wide range of chemical species, including high-boiling point compounds and high-polar compounds, and provides reliable analytical results.
In the residual solvents analysis of medicines, analysis with an aqueous solvent may be performed after analysis with a DMF solvent. With the HS-20 NX, carryover of DMF is not a problem. HS-20 NX is effective for the analysis of samples with different type of solvents or large concentration differences.



No carryover of DMF

Analysis of Elemental Impurities in Oral Drug Products Using ICPMS —The Japanese Pharmacopoeia Eighteenth Edition—

Application

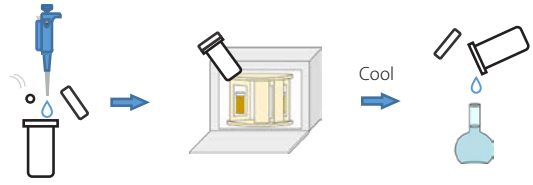
Note: The data shown is not from analyzing an oligonucleotide therapeutic.

benefits

- Robust quantitative analysis of control thresholds even when Option 1 (ICH Q3D) is used to convert PDEs to concentration limits
- Easily meets acceptance criteria for accuracy and precision for the analytical procedure used to quantify elemental impurities
- Preset methods eliminate the labor needed to establish analytical conditions and allow anyone to perform analysis with ease

Methods and Results

Sample	Oral drug products (OD gastrointestinal drug tablets) (not an oligonucleotide therapeutic)
Concentration and Quantity	Target concentration: Option 1 (quantity of drug administered per day: 10 g) was used to calculate concentration limit values converted from PDE values. Target concentration J-values were calculated by dividing concentration limit values by the dilution factor (for 250-fold dilution, refer to "Pretreatment").
Pretreatment (Figure 1)	4 mL purified water, 4 mL nitric acid, and 0.5 mL hydrochloric acid were added to about 0.2 g of the test samples, which were digested in a microwave digestion system (for about 60 min at 200 °C. (The hydrochloric acid was added to stabilize the concentration levels of Hg and certain other elements in the solution.) The digestion vessel was cooled to room temperature and then filled to 50 mL (250-fold dilution). Process blank solutions were also prepared to check the contamination level of each element during decomposition.
Analysis Conditions	High-frequency output: 1.20 kW Plasma gas flow rate: 9.0 L/min Auxiliary gas flow rate: 1.10 L/min Carrier gas flow rate: 0.85 L/min Cell gas: He Standard substances: Standard multi-element mixture solution for ICHQ3D oral drugs (Cd, Pb, As, Co, V, and Ni) and standard individual element mixture solution (for internal standard) for ICHQ3D standard mercury solution (Sc, Ga, In, and Bi).
Results	Converted concentration in test sample solid: Concentrations in solids were converted by subtracting process blank concentrations from the sample solution concentrations and multiplying the result by the dilution factor. Concentrations in the sample solution were equal to or less than the J-value for all elements. Compliance standards for pharmacopoeia quantitative testing include trueness with a spike recovery rate within 70 to 150 %, accuracy with an RSD value of 20 % or less, and a limit of quantitation that satisfies the trueness standard for concentrations of 0.5 J or less. For the analytical results in this case, the spike recovery rates were within 94 to 104 %, RSD was 3 % or less, and all results easily satisfied compliance standards. Additionally, the results confirmed that the 10 σ value was more than adequately low, at one-tenth or less of the 0.5 J criterion value. (Table 2)



(1) Place the acid and test sample in the digestion vessel

(2) Digest in a microwave digestion system

(3) Make up to a fixed volume

Figure 1 Sample Preparation Procedure

Table 1 Equipment Configuration

Instrument:	ICPMS-2050
Nebulizer:	Nebulizer DC04
Chamber:	Cyclone Chamber
Torch:	Mini-Torch
Skimmer Cone:	Nickel
Autosampler:	AS-20
Internal Standard Elements:	Online Internal Standard Kit (sample: internal standard = about 9 : 1)

Table 2 Quantitative Results

Class	Element	IDL [µg/L]	Preparation Blank Concentration [µg/L]	Sample Solution Concentration [µg/L]	Concentration in Solid Sample [µg/g]	Spiked to 50 % of the Target Concentration		Spiked to 100 % of the Target Concentration			Spiked to 150 % of the Target Concentration	
						Spiked Concentration [µg/L]	Recovery [%]	Spiked Concentration [µg/L]	Recovery [%]	RSD [%]	Spiked Concentration [µg/L]	Recovery [%]
1	^{111}Cd	0.006	N.D.	N.D.	N.D.	0.3	94	0.6	97	2.2	0.9	96
	^{208}Pb	0.001	0.005	0.029	0.006	0.3	102	0.6	103	0.95	0.9	103
	^{75}As	0.01	N.D.	N.D.	N.D.	0.9	103	1.8	100	0.74	2.7	99
	^{202}Hg	0.003	N.D.	N.D.	N.D.	1.8	96	3.6	96	0.94	5.4	97
2A	^{59}Co	0.003	N.D.	N.D.	N.D.	3	100	6	100	0.73	9	100
	^{51}V	0.02	N.D.	N.D.	N.D.	6	103	12	104	0.93	18	104
	^{60}Ni	0.1	N.D.	0.2	0.05	12	104	24	104	0.60	36	104

IDL = Standard deviation upon repeated analysis of the blank solution × 3 × Calibration curve slope
N.D. = Not Detected

Conclusion

The results confirmed that performance easily satisfied the pharmacopoeia trueness, accuracy, and quantitation limit compliance standards. In addition, the preset methods in LabSolutions ICPMS can reduce the time and trouble involved in evaluating measured masses and analysis conditions. The above results confirm that the ICPMS-2050 system is useful for analyzing elemental impurities in oral drug products.

ICP Mass Spectrometer

ICPMS-2040/2050

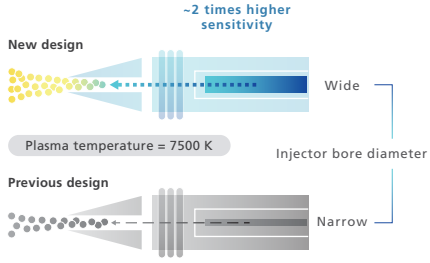
Features

The ICPMS-2040/2050 Series systems have achieved a harmonious blend of environmental-friendliness and analytical performance through the advanced proprietary Mini-Torch system. Without the need for any special options, the systems reduce measurement time, contributing to the optimization of your workflow efficiency. Moreover, the software comes with various functions, options, and maintenance information that minimize operator intervention, revolutionizing the way you work.

Product

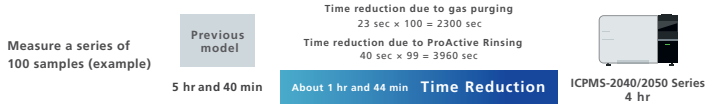
Eco Friendly yet Competent

The features of Shimadzu's mini-torch system, which consistently reduces argon gas consumption to approximately two-thirds, and can use low-purity (99.95 %) gas, remain. At the same time, optimizing the torch design decreases sample flow rate into the plasma, improving sample ionization efficiency. The new design roughly doubles sensitivity compared to previous models.



Fast at No Additional Cost

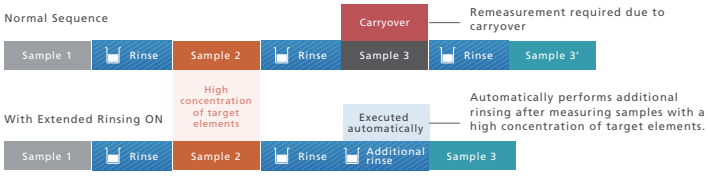
The improved gas controller features high-speed cell gas purging.
Combined with ProActive Rinsing, measurement times can be significantly shortened.



Minimal Operation Required

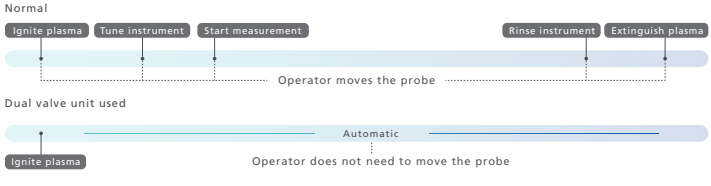
Extended Rinsing —Automatically Minimizes Carryover—

The extended rinsing function automatically performs an additional rinse sequence when a target element exceeds a predetermined upper limit. A second rinse solution can be used in the additional rinsing sequence to improve rinsing effectiveness. Consequently, carryover is eliminated to ensure high-quality data.



Dual Valve Unit —Enables Autonomous Operation after Plasma Ignition—

When using an Online Internal Standard Kit (optional), the operator must move the probe before and after data acquisition. The optional Dual Valve Unit allows seamless switching between tuning solution and rinsing solution while also automatically adding internal standard solution to samples. The entire sequence, from instrument tuning and measurement to rinsing and plasma off, is performed automatically.



Quantitation of Double-stranded DNA

– Trace Measurement Using TrayCell and Nano Stick –

Application

benefits

- Enables spectrum acquisition with ultrafast scanning at a maximum speed of 29,000 nm/min.
- The use of TrayCell (manufactured by Hellma Analytics) and Nano Stick (manufactured by SINCO) enables measurement from a sample volume of as little as 3 µL.
- The quantitative value of oligonucleotide concentration can be calculated by using the oligonucleotide quantification function in Biomethod.

Methods and Results

Sample	Double-stranded DNA
Conc., Volume	1 ~ 300 ng/mL, 2 µL
Preparation	Dilution in ultrapure water to the concentrations above.
Analytical Conditions	As shown in Table 1
Method for Measuring Double-Stranded DNA Using TrayCell	We prepared ds DNA and standard samples at 27.5, 55, 110, 220, and 440 ng/µL (ultrapure water was used as the dilution solution). For samples with unknown concentrations, we prepared the same DNA by ethanol precipitation. The TrayCell can be changed to optical path lengths of 1.0 mm and 0.2 mm by using two different lids. In this test, we used a lid with an optical path length of 1.0 mm, dropped 4 µL, and measured under the conditions listed in Table 1 (Figure 1).
Method for Measuring Double-Stranded DNA Using Nano Stick	We prepared standard and unknown samples of ds DNA as well as the above TrayCell. We also set the analysis conditions as listed in Table 1. The optical path length of the Nano Stick was 0.5 mm, and the sample volume was measured at 3 µL (Figure 2).
Results	Figures 3 and 4 show the calibration curve and UV spectrum results when measured using TrayCell and Nano Stick. Both results indicate a highly linear calibration curve. We repeatedly measured 440 ng/µL 10 times. As a result, the correlation function and CV value were calculated, and it was confirmed that the measurement was performed accurately.

Table 1 Analysis Conditions

Wavelength (calibration curve):	260 nm, 320 nm
Wavelength range:	220 nm ~ 330 nm
Scan Speed :	Low
Sampling Pitch:	1 nm

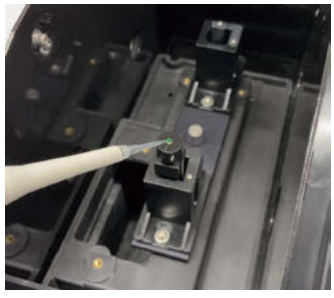


Figure 1 TrayCell Appearance

1. The sample is placed in the central silver region.

2. Set the lid on

3. Set the cell holder with Traycell

4. After measurement, wipe the sample with a Kim wipe.

Figure 2 What the Nano Stick Looks Like and How To Use It

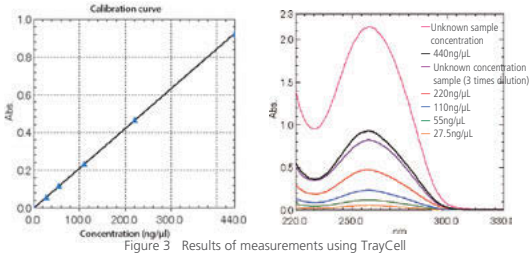


Figure 3 Results of measurements using TrayCell

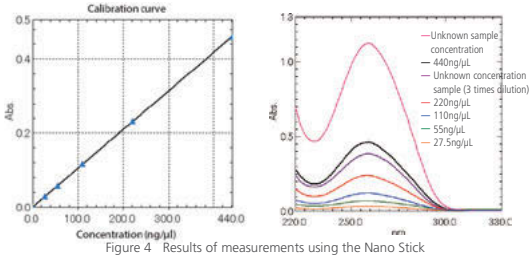


Figure 4 Results of measurements using the Nano Stick

Conclusions

This application news has shown that the UV-1900i, TrayCell, and Nano Stick can be used to make accurate and simple measurements even for small samples of the order of several µL.

UV-Vis Spectrophotometer

UV-1900i Plus

Product

Features

The UV-1900i Plus spectrophotometer features a space-saving and ergonomic hard design. It is equipped with a color touch panel and adopts a user interface (UI) that allows users to easily and quickly obtain answers. In addition, six types of measurement conditions are built in: 1. Oligonucleotide quantification, 2. Lowry method, 3. BCA method, 4. CBB (Bradford method), 5. Biuret method, and 6. UV absorption method. It also has a screen shot function on the operation panel, so you can easily extract measurement results without connecting to a PC. A 10 mm square cell requires a sample volume of approximately 4 mL, but the TrayCell or Nano Stick can be used to measure trace amounts of about 2 µL to 4 µL.

High Performance to Meet Diverse Needs

High Speed Scan	Spectra can be acquired as fast as 29,000 nm/min. Ultra-fast scanning is effective for tracking chemical reactions in a short time. In addition to the absorbance change at specified wavelengths, spectra can also be acquired in a short time with the UV-1900i Plus. Therefore, more detailed behavior can be investigated by observing spectra with the UV-1900i.
Low Stray Light	Stray light is at 0.5 % max. (198 nm), making accurate measurements possible up to the vicinity of 2 Abs even in the ultraviolet region. In addition, high-concentration samples can be quantified accurately.
High Reproducibility and Repeatability Accuracy	High repeatability of less than 0.0002 Abs reduces variation in measurement results. And it enables more accurate quantitative analysis and detection of lower concentrations of samples.
A Diversity of Measurement Modes	UV-1900i Plus features six measurement modes: Photometric, Spectral, Quantitative, Kinetics, Timecourse, and Biomethod.

Full Support for Pharmacopeia, GLP/GMP, FDA 21 CFR Part 11 and Other Regulations

Instrument Validation Functions Compliant with JP, USP, and EP	This instrument can not only run checks for nine JIS items, but also those stipulated in the Japanese Pharmacopoeia (JP), United States Pharmacopeia (USP), and the European Pharmacopoeia (EP). Naturally, the hardware is also compliant with the specifications required by each Pharmacopeia. In addition, the check conditions can be saved. As a result, once the conditions are saved, checks can be performed easily just by calling them up as needed. Check results can also be saved.
Resolution of 1 nm, the Highest in its Class	In addition to achieving a resolution of 1 nm, the highest in its class, by using a monochromator with a Czerny-Turner mounting, the UV-1900i also features a compact, bright optical system. The instrument is more than capable of meeting the wavelength resolution required in the European Pharmacopoeia.
Improved Security Functions	An external control security function has been added to provide more support for compliance with regulations. Three user authority levels, "Administrator", "Developer", and "Operator", can be set for instrument users.
Support for Regulations and Guidelines	Ensuring the integrity of data (database management), including the user management, user authority management, and data audit trails, required for compliance with FDA 21 CFR Part 11, PIC/S GMP guidelines, and other ER/ES regulations is possible. LabSolutions DB UV-Vis or UVProbe / LabSolutions DB System allows for data management and user management with a database. Compliant with ER/ES regulations, the system is optimally configured for customers using a PC. LabSolutions CS UV-Vis or UVProbe / LabSolutions CS System (Network System) is optimally configured for customers who want to manage data on a server together with LC and GC data for ER/ES compliance.

Quantitating siRNA Oligonucleotide and Confirming Molecular Weights Using a Triple Quadrupole Mass Spectrometer

Application

benefits

- High-sensitivity quantitation and molecular weight confirmation are possible with one system.
- Ease of maintenance helps minimize downtime.

Methods and Results

Sample	5′-pU CGAAGUAUUCCGCGUACG dTdT-3′ Average molecular weight: 6646.0 5′-pC GUACGCGGAAUACUUCGA dTdT-3′ Average molecular weight: 6669.0
Concentration and Quantity	1 to 10000 fmol/L, 10 µL
Pretreatment	Diluted with ultrapure water to the concentrations indicated above.
Measurement Conditions	Listed in Table 1.
Results	The calibration curves for AS-Oligo and SS-Oligo showed good linearity within the 1 fmol to 10000 fmol range (Figure 1), with a contribution rate (R^2) of 0.997 for AS-Oligo and 0.995 for SS-Oligo. The limit of detection was 1 fmol and the lower limit of quantitation was 5 fmol. The sensitivity of MRM (multiple reaction monitoring) analysis exceeded SIM analysis. A SIM chromatogram from a mixture solution with 100 fmol each of SS-Oligo and AS-Oligo is shown in Figure 2. Using ion pair chromatography, elution times were 6.88 minutes and 6.94 minutes, respectively, with a 0.3 separation R value. Ions with 4 to 9 charges were detected in the mass spectra (Figures 3 and 4) for both samples. Results from using LabSolutions multi-charged ion analysis functionality to calculate molecular weights resulted in estimated AS-Oligo and SS-Oligo molecular weights of 6645.0 and 6667.2, respectively, with an error factor of 1.0 Da and 1.8 Da from the average molecular weights.

Table 1 Measurement Conditions

[HPLC conditions] (Nexera)	
Column:	Commercial C18 column(100 mm×2.1 mmI.D., 1.7 µm)
Mobile Phases:	A)200 mM HFIP*1 and 7.5 mM TEA*2/water B)Methanol
Gradient Program:	B conc. 4 %(0 min) — 20 %(8.0 min)
Flow Rate:	0.2 mL/min
Column Temp.:	75 °C
Injection Volume:	10 µL
[MS conditions] (LCMS-8060)	
Ionization:	ESI(Negative mode)
Probe Voltage:	-3 kV
Mode:	Q3scan(*m/z*500-1800) SIM *m/z* 1666.0(SS), *m/z*1659.9(AS)
Nebulizing Gas Flow:	3.0 L/min
Drying Gas Flow:	5.0 L/min
Heating Gas Flow:	15.0 L/min
DL Temp.:	250 °C
Heat Block Temp.:	500 °C
Interface Temp.:	350 °C

*1 1,1,1,3,3,3-Hexafluoro-2-propanol
*2 Triethylamine

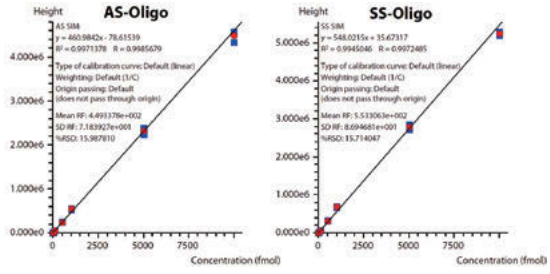


Figure 1 Calculation Curves Obtained from SIM Chromatograms

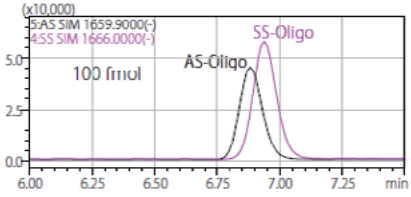


FIgure 2 SIM Chromatogram

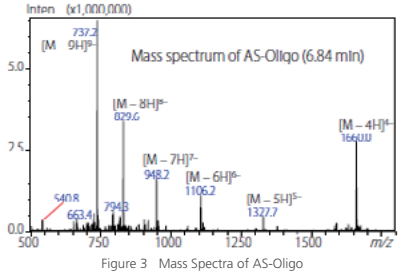


Figure 3 Mass Spectra of AS-Oligo

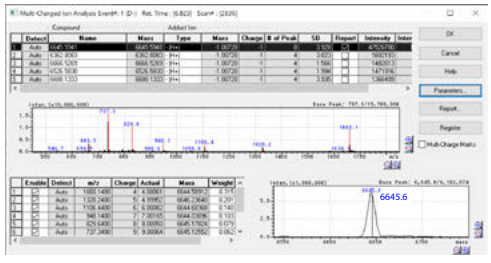


Figure 4 Spectrum from Multi-Charged Ion Analysis of AS-Oligo

Conclusions

Using an LCMS-8060 triple quadrupole mass spectrometer for SIM quantitative analysis of siRNA oligonucleotides resulted in a mass spectrum with low resolution, but the multi-charged ion analysis functionality was able to confirm molecular weights within an error range of several Da.

Triple Quadrupole LC-MS/MS

LCMS-8060RX

Product

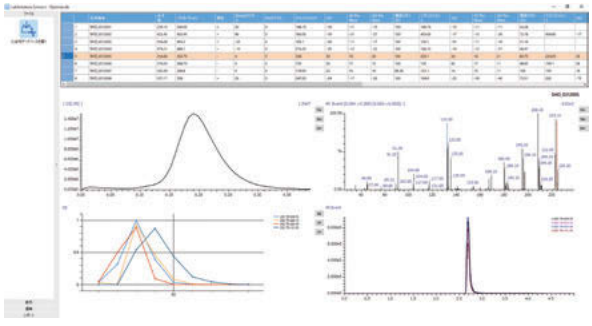
Features

LCMS-8060RX is a triple quadrupole mass spectrometer that combines high speed and high sensitivity.The mass range is *m/z* 2 ~ 2000, and molecular weight estimation of oligonucleotide therapeutics is possible using the deconvolution function of LabSolutions LCMS software.
Triple-quadrupole mass spectrometers, which have high sensitivity and a wide dynamic range, are commonly used for quantitative analysis, such as for pharmacokinetic analysis of drugs, but also offer qualitative capabilities. Precise mass spectrometry techniques such as MALDI-TOF and Q-TOF LC/MS are frequently used for accurate mass molecular weight determination.

Software Solutions from Acquisition to Data Review
LabSolutions Connect MRM / LabSolutions Insight

Automatic optimization of MS conditions

MRM parameters (precursor ion *m/z*, product ion *m/z*, voltages) and ion source parameters (gas flow rate, temperature) are automatically optimized. Just one round of comprehensive optimization maximizes sensitivity, taking into consideration polarity, adduct ions, charge number, etc. The results of this process can be viewed on a graph using the data browser function. The MRM optimization results screen simultaneously displays a chromatogram, a spectrum, and each voltage. From the MRM optimization results screen, check how the signal intensity changes with variations in each parameter.



MRM optimization
Review screen for MRM optimization simultaneously displays precursor ion, product ion, and voltage results..

Ion source optimization
Review screen for source optimization graphically displays the successive results of each parameter modification.

Short Oligonucleotide Analysis Using Supercritical Fluid Chromatography

Application

benefits

- Enables the separation of phosphorothioate-modified short-chain oligonucleotide impurities.
- In some cases, using SFC can provide different selectivity from LC.
- Using preparative SFC evaporates the carbon dioxide, one of the solvents, which simplifies post-treatment.

Methods and Results

Sample	5′-ToToToT-3′ 5′-TsToToT-3′ 5′-TsToTsT-3′ 5′-TsTsTsT-3′ T: Thymidine, o: phosphodiester linkage, s: phosphorothioate linkage
Concentration and Quantity	100 nmol/mL, 5 µL
Analysis	Samples were dissolved in methanol that contains 5 % water and analyzed using the measurement conditions listed in Table 1 to compare the retention characteristics of each column.
Results	The Diol II column was able to separate oligonucleic acids with different PS concentrations. SIL II and HyP columns separated or partially separated the acids. The PBT column did not retain the oligonucleic acids, whereas the PVP, triazole, and NH2 columns all did not elute the sequences within the given time. The results showed that retention characteristics varied depending on the column.

Table 1 Analytical conditions for Figure 1

System:	Nexera UC
Column:	Shim-pack UC-Diol II, SIL II, HyP, Triazole and NH_2 (150 mm×2.1 mm I.D., 3 µm) Shim-pack UC-PBT and PVP(150 mm×3.0 mm I.D., 3 µm)
Temperature:	35 °C
Injection volume:	5 µL
Mobile phases:	A) CO_2, B)50 mmol/L ammonium formate in methanol and water (95:5, v/v) A/B=50:50
Flow rate:	1.0 ml/min
Detection:	260 nm (PDA coupled to a high-pressure cell)

Table 2 MS conditions

System:	LCMS-9030
Polarity:	Negative
Interface temp:	350 °C
Nebulizer gas:	3.0 L/min
Heating gas:	10.0 L/min
Drying gas:	10.0 L/min
DL temp:	250 °C
Heat block temp:	400 °C
Interface voltage:	-3.5 kV
TOF-MS:	*m/z* 150-2000

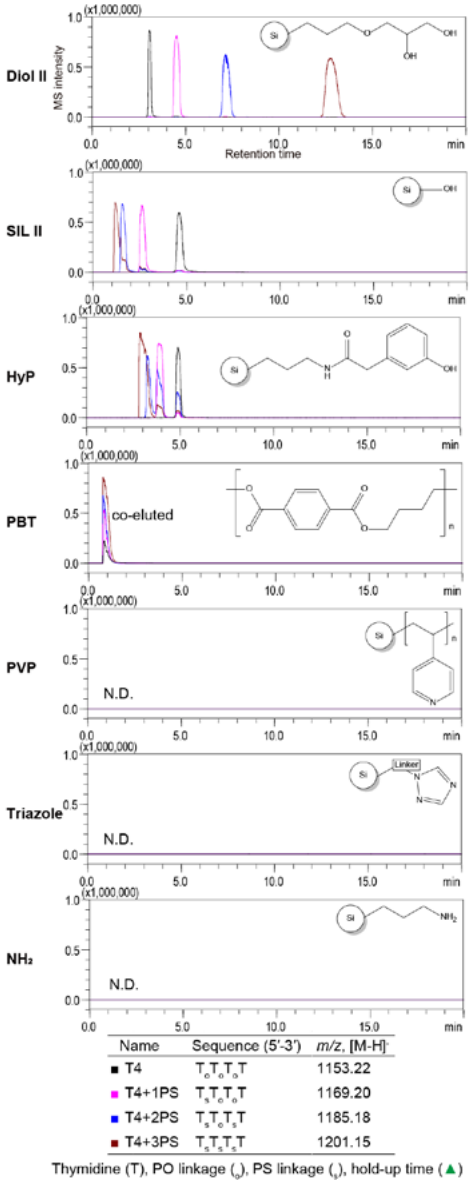


Figure 1 Overlayed EICs of T4s with different PS contents

Conclusions

These results indicate that SFC can be used to analyze short-chain oligonucleic acids by optimizing the column and other analysis conditions.

Supercritical Fluid Extraction / Supercritical Fluid Chromatograph

Nexera UC

Product

Features

Reverse-phase ion pair chromatography is typically used to analyze oligonucleic acids, but some impurities that have a similar structure to the target substance can make separation difficult. Therefore, development of a separation method with different selectivity from the existing method is anticipated.
Supercritical fluid chromatography (SFC) is an analysis method that uses carbon dioxide as the mobile phase at a temperature and pressure that are both above the critical point. Because carbon dioxide has lower viscosity and higher diffusivity than regular liquids, reports have indicated that it has higher separation and special selectivity characteristics. Meanwhile, the low polarity of mobile phases that contain carbon dioxide makes them unsuitable for analyzing highly polar compounds. However, in recent years, it has become possible to analyze highly polar compounds, such as peptides and nucleotides, by selecting an appropriate stationary phase and modifier composition for mixing with the carbon dioxide.

Nexera UC systems can automatically extract target components from solid samples using supercritical CO_2 fluid and then seamlessly analyze and separate them directly in an online process.
The unique gas-liquid separation technology used offers both easy operation and preparative purification with high recovery rates by inhibiting any drop in recovery rates due to eluate dispersion during CO_2 evaporation. This achieves high recovery rates even for volatile components.

Nexera UC/s UHPLC / SFC Switching System

This system can switch automatically between SFC analysis and UHPLC analysis and make measurements on a single sample in each separation mode. It enhances user-friendliness by allowing the investigation of separation conditions and performing reverse-phase high-speed analysis using a single system. Shimadzu also provides a kit to upgrade from your current UHPLC system to the UHPLC/SFC switching system.

Analysis of Deaminated Oligonucleotides Using Supercritical Fluid Chromatography

Application

benefits

- Using optimized modifier containing octylamine, 18-mer modified oligonucleotides were detected.
- As a result of optimization of analytical conditions for the separation of deaminated oligonucleotides, 10- and 18-mer target oligonucleotides and their deaminated products were able to be separated.

Methods and Results

Optimization of analytical conditions for separating deaminated oligonucleotides from desired oligonucleotides

To evaluate the separation performance of deaminated oligonucleotides with SFC, 2'-MOE-modified oligonucleotides (Figure 1) and their deaminated sequences were used to investigate the column oven temperature and the type of modifier additive. Since the choice of alkyl amine greatly affected the separation ability in IP-RPLC, same way of investigation was done on SFC (Figure 2). First, primary, secondary, and tertiary alkylamines in same carbon number (hexylamine, dipropylamine, and triethylamine) were investigated. No peak was detected for triethylamine, a broad peak was detected for dipropylamine, and a sharp peak was detected for hexylamine. Since hexylamine is the most hydrophobic of the three alkyl amines, the hydrophobicity of the alkyl amine was thought to be involved in the peak shape. When dibutylamine, a secondary amine with greater hydrophobicity than hexylamine, was employed, a broad peak was detected. This suggests that long-chain, primary amines would be effective for obtaining good peak shapes. Finally, octylamine was continuously used because it provided the best peak shapes and the highest resolutions among the evaluated alkyl amines.

Table 1 Analytical conditions for Figure 2

System :	Nexera UC
Column :	Shim-pack UC-Diol II (150 mm×4.6 mm I.D., 3 µm*1)
Column oven temp. :	50 °C
Injection volume :	1 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, v/v)
Mobile phases :	A) CO_2 B) 50 mmol/L alkylamine and 50 mmol/L acetic acid in methanol and water (95:5, v/v)
B conc :	30 % (0–5 min), 30–60 % (5–30 min), 60 % (30–35 min), 30 % (36–40 min)
Flow rate :	1.0 mL/min
Back pressure :	10 MPa, 50 °C
Detection :	PDA 260 nm

*1 Custom-made

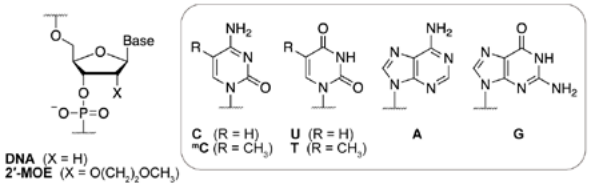
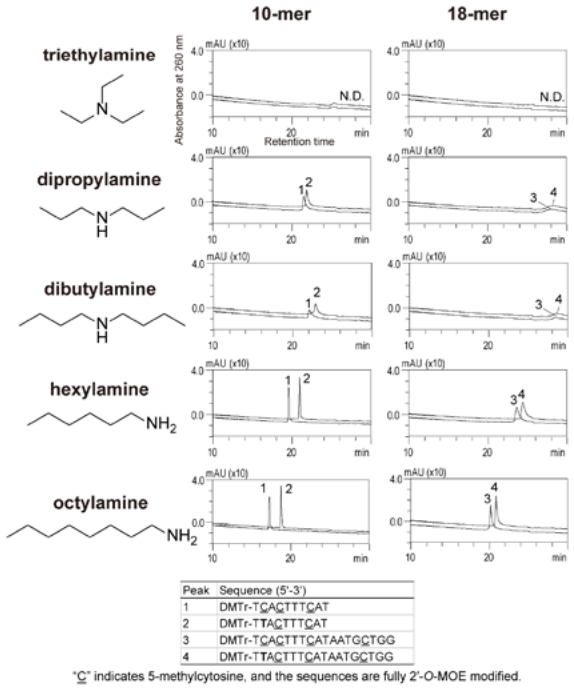


Figure 1 Structures of DNA and 2'-MOE-modified oligonucleotides



Peak	Sequence (5'-3')
1	DMTr-TCACTTTCAT
2	DMTr-TTACTTTCAT
3	DMTr-TCACTTTCATAATGCTGG
4	DMTr-TTACTTTCATAATGCTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-O-MOE modified.

Figure 2 Chromatographic separations of target (peak 1 and 3) and deaminated (peak 2 and 4) sequences using different alkyl amines inmodifiers

Evaluation for separation behavior of deaminated oligonucleotides

The separations of target oligonucleotides and sequences with different positions of deamination were investigated (Fig. 3). In the case of DMTr-on 10mer and -off 10-mer, the target sequence was able to be separated from deaminated sequences. In the case of IP-RPLC, some deaminated sequences were separated from DMTr-on 10-mer, but sequence d was co-eluted. IP-RPLC showed excellent separation of both DMTr-off 10-mer and 18-mer sequences. These results suggest that SFC and IPRPLC showed different retention behaviors in oligonucleotide analysis.

Table 2 Analytical conditions of SFC for Figure 3

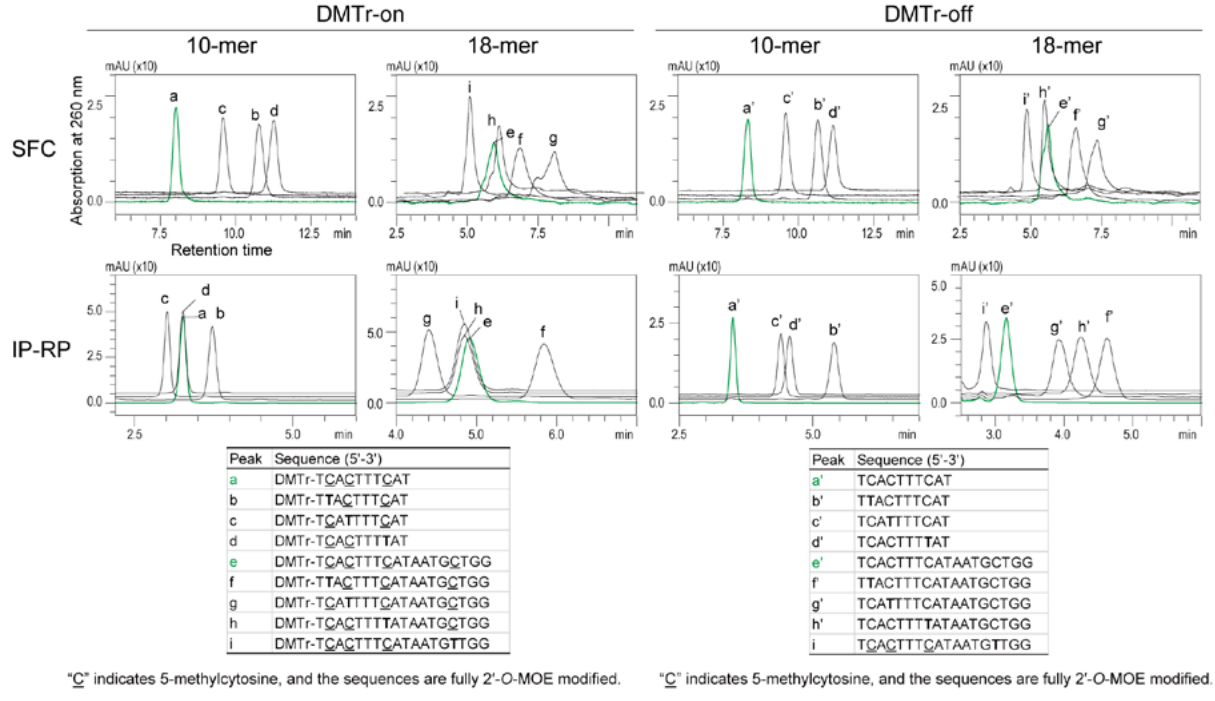
System :	Nexera UC
Column :	Shim-pack UC-Diol II (150 mm×4.6 mm I.D., 3 µm*1)
Column oven temp. :	50 °C
Injection volume :	1 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, v/v)
Mobile phases :	A) CO_2 B) 50 mmol/L octylamine and 50 mmol/L acetic acid in methanol and water (95:5, v/v)
B conc :	40 % (DMTr-on and -off 10-mer), 45 % (DMTr-on and -off 18-mer)
Flow rate :	1.0 mL/min
Back pressure :	10 MPa, 50 °C
Detection :	PDA 260 nm

*1 Custom-made

Table 3 Analytical conditions of IP-RPLC for Figure 3

System :	Nexera XS
Column :	Shim-pack Scepter C18-120 (150 mm×4.6 mm I.D., 3 µm*1)
Column oven temp. :	50 °C
Injection volume :	1 µL of 100 µmol/L oligonucleotide dissolved in methanol and water (95:5, v/v)
Mobile phases :	A) 10 mmol/L propylamine (pH 9.6, adjusted with acetic acid) in water B) acetonitrile
B conc :	30 % (0–5 min), 30–60 % (5–30 min), 60 % (30–35 min), 30 % (36–40 min)
Flow rate :	1.0 mL/min
Back pressure :	10 MPa, 50 °C
Detection :	PDA 260 nm

*1 P/N 227-31016-05



Peak	Sequence (5'-3')
a	DMTr-TCACTTTCAT
b	DMTr-TTACTTTCAT
c	DMTr-TCATTTTCAT
d	DMTr-TCACTTTTAT
e	DMTr-TCACTTTCATAATGCTGG
f	DMTr-TTACTTTCATAATGCTGG
g	DMTr-TCATTTTCATAATGCTGG
h	DMTr-TCACTTTTATAATGCTGG
i	DMTr-TCACTTTCATAATGTTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Peak	Sequence (5'-3')
a'	TCACTTTCAT
b'	TTACTTTCAT
c'	TCATTTTCAT
d'	TCACTTTTAT
e'	TCACTTTCATAATGCTGG
f'	TTACTTTCATAATGCTGG
g'	TCATTTTCATAATGCTGG
h'	TCACTTTTATAATGCTGG
i	TCACTTTCATAATGTTGG

"C" indicates 5-methylcytosine, and the sequences are fully 2'-*O*-MOE modified.

Figure 3 Chromatographic separations of target and deaminated sequences using SFC and IP-RPLC
Target (peak a, e, a', and e', green lines) and corresponding deaminated (peak b-d, f-i, b'-d', and f'-i', black lines) sequences

<Acknowledgments>
We would like to thank Prof. Satoshi Obika and Prof. Takao Yamaguchi (Graduate School of Pharmaceutical Sciences, Osaka University) for their guidance. This research was partially supported by AMED under Grant Number JP21ae0121022, JP21ae0121023, JP21ae0121024 (Project leader: Satoshi Obika).

Inert Column

Shim-pack Scepter Claris

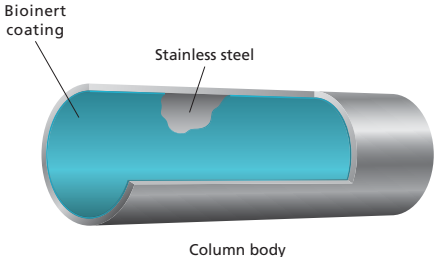
Product

benefits

- A bioinert coating inhibits adsorption.
- A hybrid organosilica material provides high resistance to high-pH mobile phases.
- An extensive line of stationary phases is available, such as wide-pore packing materials suitable for medium and large molecules.

Features

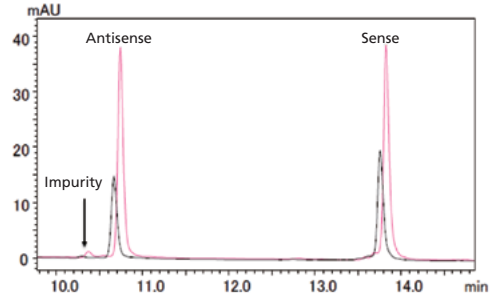
Bioinert Coating Provides Outstanding Low-Adsorption Characteristics



Shim-pack Scepter Claris columns feature a column body with a bioinert coating and a Scepter packing material.
The column offers excellent resistance to the adsorption of compounds prone to adsorbing to metals, especially nucleic acids.

Example of siRNA Analysis

Shim-pack Scepter (stainless body) and Shim-pack Scepter Claris (body with inert coating) columns were compared for analyzing synthetic siRNA. This showed that sensitivity for antisense and sense chains differed by more than a factor of 2. Moreover, a low-concentration impurity peak was detectable using the Claris column but not with the stainless steel body.

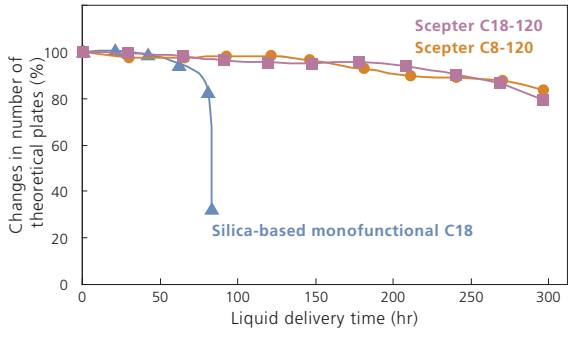


Extensive Variety of Stationary Phases Enables a Wide Range of Selectivity for Analysis

Shim-pack Scepter series columns are available with an extensive line of stationary phases with different separation performance, such as C4, C8, or C18 linear alkyl chains, phenyl, PFPP, and diol phases. C4 and C18 stationary phases are also available as wide-pore packing materials optimized for medium and large molecules. They offer excellent chromatographic performance for analyzing nucleic acids, proteins, and so on.

Durability for Using High-pH Mobile Phases

Column performance was evaluated after flushing them for a certain period with a mobile phase that has a higher pH than used for nucleic acid analysis (pH 11.5) to determine whether performance dropped. A basic mobile phase resulted in a sharp drop in column performance for the typical silica C18 column, but the Shim-pack Scepter packed with a hybrid organosilica base material showed high resistance to the basic mobile phase.



Mobile phase : 50 mmol/L triethylamine (pH 11.5)/methanol = 90/10 (v/v)
Flow Rate : 1.0 mL/min
Column Temp. : 40 °C
Sample : Benzyl alcohol

High-Quality LC and LC–MS Vials

Shim-vial H glass

Product

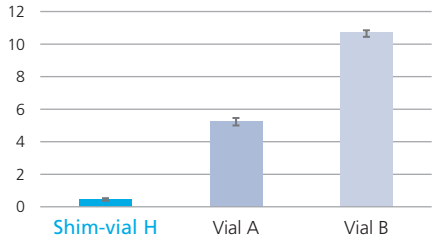
benefits

- Each vial inspected visually to ensure thorough quality control.
- Proprietary treatment (Shim-vial H glass) provides extreme inhibition of adsorption by basic compounds.
- Inhibition of Na elution provides consistent results even for samples vulnerable to pH changes.

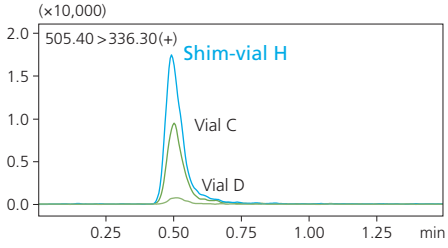
Features

Metal Ion-Exclusion Inhibits Adsorption by Basic Compounds

Presumably, the metal ions (Na) contained in the glass affect the adsorption of basic compounds. Due to a special treatment process during forming, Shim-vial H achieve exceptional low-adsorption performance by thoroughly excluding Na ions. Even the Shim-vial S, made with a simplified version of part of the treatment process, offers excellent low-adsorption performance.



Na Elution Quantity (µg/mL)



Chlorhexidine (100 µg/L, 1 µL injection)

The Smallest Possible Volumes Are Achieved with High-Accuracy Machining Techniques

Our small-volume vials with their unique shape are made of the same materials and receive the same cleaning treatment as the 1.5 mL vials. They suppress adsorption more than other small-volume inserts by limiting the contact surface between the sample and the container. In addition, the bottom surface of the vial is hollowed out in order to increase thermal conductivity.

Residual volume: Approx. 10 µL

When using it, adjust the needle stroke parameter to be 15 mm higher than for the 1.5 mL vials.
For example: Shimadzu SIL-40 series: 32 mm or less

Vial Selection by Application

Our lineup includes Shim-vial H, which enables trace-quantity analysis of basic compounds prone to adsorption by glass, thanks to advanced low adsorption treatment, and the cost-effective Shim-vial S.

For high-sensitivity analysis **Shim-vial H**

- ppt order high-sensitivity analysis (LC–MS)
- Analysis of compounds prone to adsorption (LC–MS and LC)
- Analysis with a focus on repeatability (QA/QC)
- Analysis requiring a low background

For routine analysis **Shim-vial S**

- Analysis of general compounds (LC–MS and LC)
- ppm to ppb order high-sensitivity analysis
- Analysis with a focus on repeatability (QA/QC)
- Analysis requiring a low background

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