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Application News

LabSolutions™ MD : Software for Efficient Method Development based on Analytical Quality by Design

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

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User 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.

■ Introduction

Nucleic acid drugs, such as antisense oligonucleotides, exert their effect by interacting with targets (genes and proteins) inside and outside of cells. Nucleic acid drugs are produced through chemical synthesis, but the synthesis process can introduce impurities such as shorter and longer length of products and protection groups. Therefore, proper separation of the target oligonucleotide is required.

For LC separation, one commonly used mode is reversed-phase ion-pair chromatography (RP-IP). The separation patterns obtained with RP-IP chromatography can vary depending on the concentration of the ion pair reagent and the composition of organic solvent. In addition, the separation behavior can differ based on the length of products, nucleobase, and the presence of modifications. Therefore, it is important to optimize the separation for each sequence of oligonucleotides. This article describes how to achieve the optimal separation of oligonucleotides and related impurities efficiently by utilizing LabSolutions MD, a dedicated software for supporting method development, through initial screening and optimization phase respectively.

■ Sample Information

A target oligonucleotide and five related impurities that have different sequences are used as a model sample of synthetic antisense oligonucleotide (Table 1). Full length product (FLP), deletion sequences of n-1(3′), n-1(5′), and n-3 as shortmers, addition sequence of n+1 as longmer, and PO (modified from phosphorothioate to phosphate diester at 5′) are prepared as a sample mixture.

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*-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

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

■ Initial Screening of Mobile Phase

For initial screening (analytical conditions in Table 2), the parameters that have a large effect on separation such as the concentration of HFIP and ion-pair reagent (triethylamine: TEA) in the aqueous mobile phase, and the mixture ratio of acetonitrile and methanol in the organic solvent were considered. Specifically, two different concentration levels of HFIP at 100 and 200 mmol/L, four different concentration levels of TEA at 5, 10, 15, and 20 mmol/L, and three different levels of acetonitrile ratio at 0, 50, and 100 % in organic solvent were evaluated (in total of 24 patterns (2×4×3)) with the aim of finding the combination for the optimal separation of target oligonucleotide and impurities. LabSolutions MD can quickly and easily generate analysis schedule by setting the parameters such as several types of mobile phases and column oven temperature (steps (1) to (5) in Fig. 1). In addition, mobile phase blending function automatically prepares the mobile phases that have different concentration of HFIP and TEA as well as the mixture ratio of acetonitrile and methanol by simply clicking the mobile phases to use (step (1) in Fig. 1) for automated screening. This significantly reduces the amount of work required for manual preparation and prevents human errors for preparation.

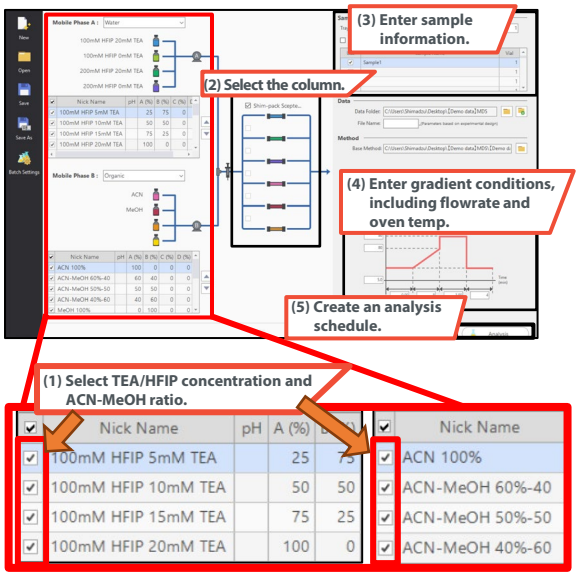

Fig. 1 Steps for Creating Analysis Schedule

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
Flowrate:	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
*2 1,1,1,3,3,3-hexafluoro-2-propanol
*3 Triethylamine

■ Results of Initial Screening of Mobile Phase

Chromatograms (FLP and each impurity) measured under different conditions of the concentration of HFIP and TEA as well as different mixture ratio of acetonitrile and methanol are shown in Fig. 2.

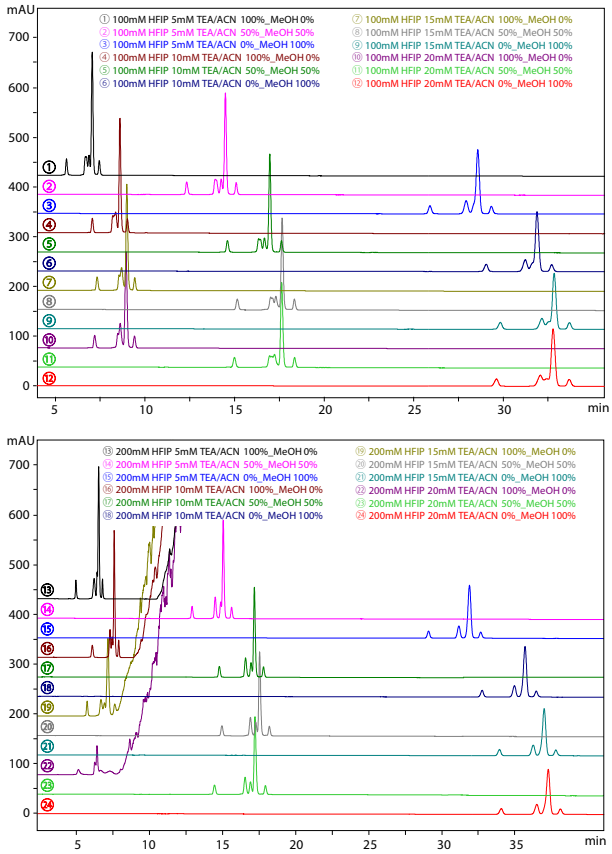

Fig. 2 Chromatograms Obtained by Mobile Phase Screening
100 mmol/L HFIP (Upper) and 200 mmol/L HFIP (Lower)

The result of mobile phase screening shows that the concentration of HFIP and TEA in the aqueous mobile phase, and the mixture ratio of acetonitrile and methanol in the organic solvent have a large effect on separation of FLP and related impurities. In addition, baseline fluctuations were observed at the condition of 200 mmol/L HFIP with 100 % acetonitrile ratio (⑬, ⑯, ⑲, and ㉒ in Fig. 2). This could affect the peak shape and quantification of each sample.

■ Quickly Find Optimal Condition

Because screening generates as many chromatograms as the number of analysis schedule, they must be evaluated to determine which one is the optimal. Checking all chromatograms manually is troublesome and time consuming. LabSolutions MD can quickly and easily find optimal condition using equation (Eq. 1) below to quantitatively evaluate the chromatographic separation.

$$(\text{Evaluation Value}) = P \times (Rs_1 + Rs_2 + \ldots + Rs_{P-1}) \qquad \text{(Eq. 1)}$$

Evaluation Value is calculated as the number of peaks detected (P) multiplied by the sum of resolution factor (Rs) for all peaks. Fig. 3 shows Evaluation Value obtained through mobile phase screening and listed in order from the highest to the lowest. It indicates that 100 mmol/L HFIP, 10 mmol/L TEA, and 50 % acetonitrile ratio provides the highest value as optimal condition. (chromatogram ⑤ in Fig. 2, enlarged in Fig. 4). The result also shows that n-1(5′) and n-1(3′) are not well separated. That is presumably because n-1(5′) and n-1(3′) have the similar structure with the same length.

Next, for the optimization phase, the parameters of column oven temperature and gradient program are considered to further improve the separation.

MPA Nick Name	MPB Nick Name	Evaluation Value
100mM HFIP 10mM TEA	ACN 50%_MeOH 50%	54.074
100mM HFIP 15mM TEA	ACN 50%_MeOH 50%	53.77
100mM HFIP 5mM TEA	ACN 50%_MeOH 50%	52.477
100mM HFIP 20mM TEA	ACN 50%_MeOH 50%	51.919
200mM HFIP 20mM TEA	ACN 50%_MeOH 50%	47.016
200mM HFIP 15mM TEA	ACN 50%_MeOH 50%	46.926
200mM HFIP 10mM TEA	ACN 50%_MeOH 50%	46.836
200mM HFIP 5mM TEA	ACN 50%_MeOH 50%	45.719
100mM HFIP 10mM TEA	ACN 100%_MeOH 0%	38.822
200mM HFIP 10mM TEA	ACN 100%_MeOH 0%	37.732

Fig. 3 Ranking of Each Condition by Evaluation Value
(Top 10 Chromatograms Listed from the Highest to the Lowest)

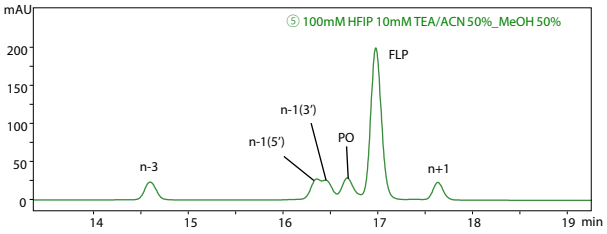

Fig. 4 Chromatogram of Highest Evaluation Value
(Enlarged Chromatogram of Fig. 2 ⑤)

■ Optimization Phase

Based on the optimal condition of mobile phase at initial screening phase, analytical condition is further optimized for the separation of FLP and related impurities by changing the parameters of acetonitrile ratio in the organic solvent (40, 50, and 60 %), column oven temperature (55, 60, and 65 °C), and initial concentration of gradient program (6, 7, and 8 %). The obtained chromatograms are shown in Figs. 5 to 7. The results show that the higher the acetonitrile ratio, column oven temperature, and initial concentration are, the better the resolution of each peak tends to be.

Next, to visualize the resolution of each peak by design space, FLP and impurities were tracked through all the data.

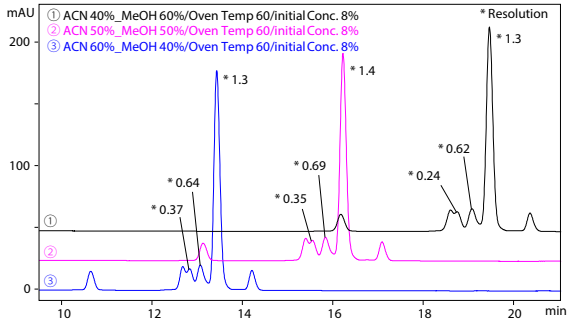

Fig. 5 Chromatograms with Different Acetonitrile Ratio
40 % (①), 50 % (②), and 60 % (③)

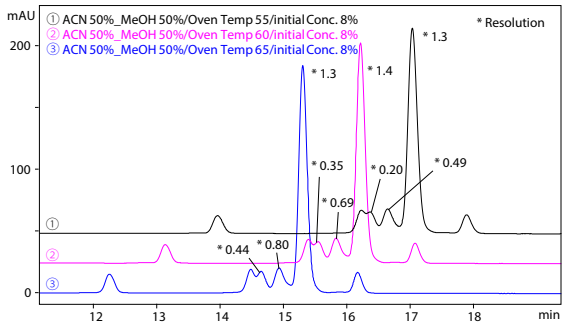

Fig. 6 Chromatograms with Different Column Oven Temperature
55 °C (①), 60 °C (②), and 65 °C (③)

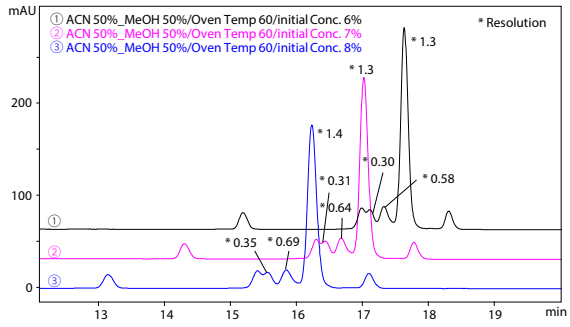

Fig. 7 Chromatograms with Different Initial Concentration
6 % (①), 7 % (②), and 8 % (③)

■ Automated Peak Tracking by LCMS-2050

LabSolutions MD can automatically identify and track each peak through all the data by simply selecting the parameters to use for tracking in a drop-down list (red square in Fig. 8). Even though retention time is changed with the different LC parameters, LabSolutions MD can utilize several parameters such as elution order of peaks, peak area, molecular weight (estimated by deconvolution of mass spectra) and so on for accurate peak tracking. This feature strongly improves the operational efficiency by reducing the risk of human errors during peak tracking.

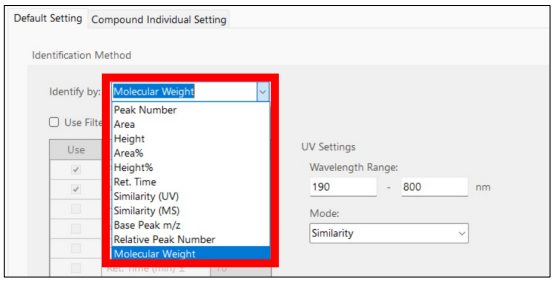

Fig. 8 Parameter Setting for Peak Tracking with LabSolutions MD

LC chromatograms obtained at the column oven temperature of 60 °C, initial concentration of gradient program of 8 %, and acetonitrile ratio of 50 % and 60 %, along with molecular weights for FLP and impurities are shown in Fig. 9. UV spectra for each impurity are shown in Fig. 10. The similarity between UV spectra of FLP, n-3, n-1(5′), n-1(3′), PO, and n+1 are more than 0.99 or higher, suggesting that peak tracking based on UV spectrum would be difficult. In contrast, LabSolutions MD enables peak tracking based on molecular weights acquired by LCMS-2050 for accurate identification of compounds that have similar UV spectra (Fig. 9). The estimated molecular weights were FLP : 7167 (theoretical MW : 7168), n-3 : 5985 (theoretical MW : 5986), n-1(5′) : 6772 (theoretical MW : 6774), n-1(3′) : 6773 (theoretical MW : 6775), PO : 7151 (theoretical : 7152), and n+1 : 7561 (theoretical MW : 7563). These results show small mass errors from the theoretical values, indicating accurate peak tracking is enabled by LCMS-2050. By deconvoluting mass spectra, molecular weights can be easily estimated. This can be utilized for confirming the molecular weights of known compounds as well as the approximate estimation of the molecular weights of unknown impurities.

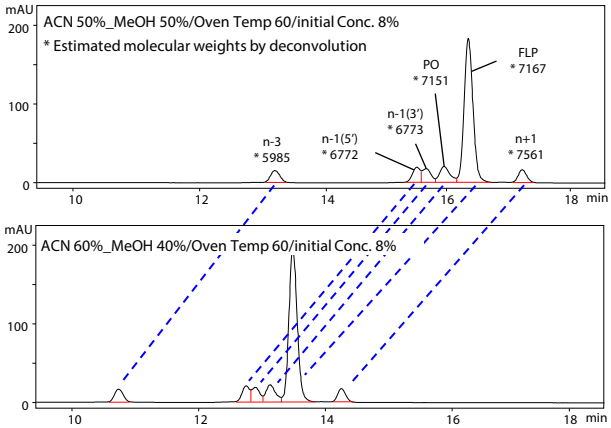

Fig. 9 LC Chromatograms at Column Oven Temperature 60 °C, Initial Concentration 8 %, and Acetonitrile Ratio 50 % (Upper) and 60 % (Lower)
(Dashed lines indicate tracking based on molecular weights.)

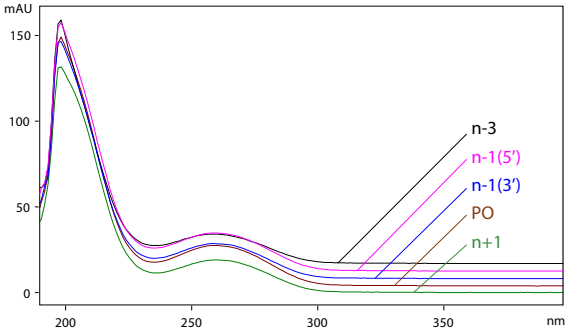

Fig. 10 UV Spectra of Impurities

Next, by visualizing resolution of FLP and each impurity with design spaces, the optimal condition that provides the best resolution and robustness is investigated.

■ Design Space Evaluation for Optimal Condition

Design spaces of resolution of FLP and each impurity were shown (Fig. 11). The vertical line shows acetonitrile ratio and the horizontal line shows column oven temperature. The red region indicates higher resolution, and the blue region indicates lower resolution. By visualizing resolution through design space, it became evident that the higher the column oven temperature is, the better the resolution of each peak gets, while optimal ratio of acetonitrile is compound-related.

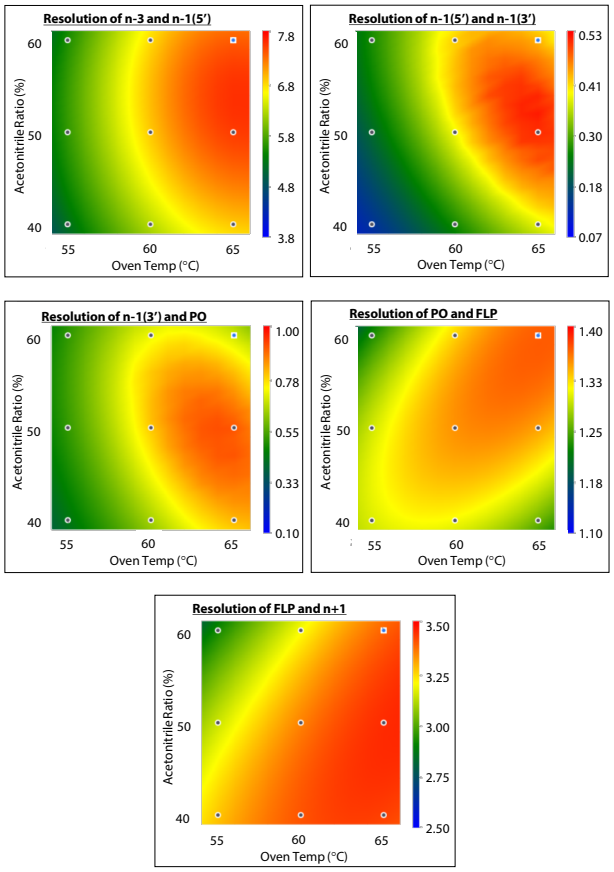

Fig. 11 Design Space for Resolution of FLP and Related Impurities
(Initial concentration is 8 %.)

LabSolutions MD can automatically search for the optimal condition that meets several criteria by overlaying design spaces. For example, Fig. 12 shows the area of the analytical condition that maximizes the resolution of n-1(5′) and n-1(3′) under the condition of the resolution of n-1(3′) and PO > 0.7, and the retention time of last eluting peak (n+1) < 16 min. The region enclosed by the green line is where the resolution of n-1(3′) and PO < 0.7, the region enclosed by the orange line is where the retention time of last eluting peak (n+1) > 16 min, and point A (in red circle) in the remaining region (shown by the black hatching) is the condition searched automatically that provides the maximum resolution of n-1(5′) and n-1(3′). By overlaying design spaces of resolution and RT of last eluting peak, optimal condition that provides enough resolution and shorter analysis time can be easily found.

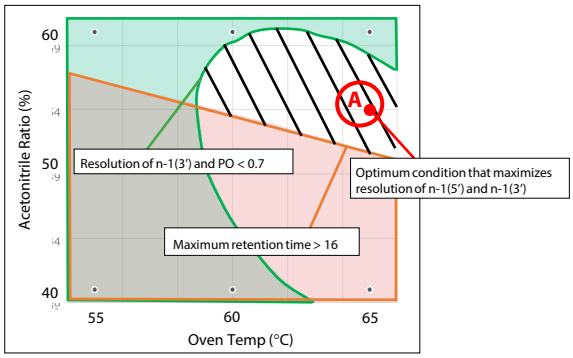

Fig. 12 Overlay of Design Spaces of Resolution and Retention Time of Last Eluting Peak

■ Chromatogram at Optimal Condition

The chromatogram obtained at optimal condition (point A) is shown in Fig. 13. It shows that the resolution of n-1(3′) and PO >0.7, and retention time of last eluting peak (n+1) < 16, which successfully satisfies the criteria for optimization. It is difficult to have sufficient separation between n-1(5′) and n-1(3′) due to their very similar structure but LabSolutions MD can maximize the resolution without depending on user's experience.

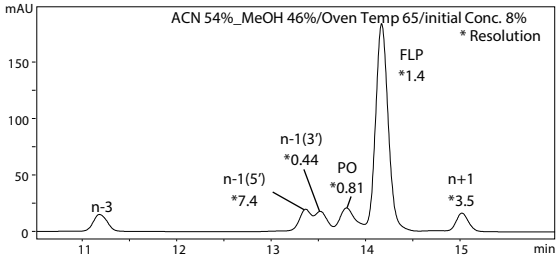

Fig. 13 Chromatogram at Optimal Condition
(100 mmol/L HFIP and 10 mmol/L TEA)

■ Conclusion

The separation pattern of oligonucleotide is different depending on the concentration of HFIP and TEA in the aqueous mobile phase, and the ratio of acetonitrile and methanol in the organic solvent in addition to column oven temperature and initial concentration of gradient program. The separation behavior can differ based on the structure of oligonucleotide such as length, nucleobase, and the presence of modifications. Therefore, it is required to optimize the separation for each sequence of oligonucleotide individually. On the other hand, a number of analyses and data processing for the optimization of analytical condition is a time-consuming challenge. LabSolutions MD can automate the entire workflow, including the generation of 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, Shin-pack Scepter Claris, and LCMS-2050 strongly improves the efficiency of the overall workflow of method development of oligonucleotides.

■ Acknowledgments

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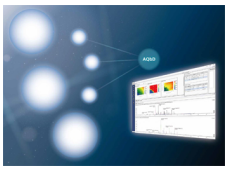

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