Application Note

Agilent
Trusted Answers

Assessment of Linearity Performance on an LC/MS System Operated in Negative-Ion Mode

Using the Agilent LCMS OQPV Negative Mode Standard kit

Authors

Ace G. Galermo, Matt Brittain, Cedric Le Doeuff, Shawn Ehlers-Cheang, Jesse Stone, and Tom Rice
Agilent Technologies, Inc.

Abstract

Operational qualification (OQ) of a mass spectrometry system is routinely performed in positive-ion mode to ensure the instrument is accurately operating for quantitative analyses. However, this introduces a pain point for users with dedicated liquid chromatography/mass spectrometry (LC/MS) instruments for oligonucleotide analyses as the system is often contaminated with ion-pairing reagents that can affect positive-ion mode OQ such as 1,1,1,3,3,3-hexafluoro-2-propanol-triethylamine (HFIP) and triethylamine (TEA). To address this, Agilent has developed the LCMS OQPV Negative Mode Standard kit (p/n 5191-4567) to enable OQ to be performed exclusively in negative-ion mode, eliminating the need for tedious cleansing of ion-pair reagents from MS systems dedicated to negative-ion mode. This technical note describes the use of the Agilent LCMS OQPV Negative Mode Standard kit for the development of a 15-minute reverse-phase C18-based LC/MS method for the assessment of calibration curve linearity and quantitative reproducibility of an ultrahigh-performance LC (UHPLC) system, the Agilent 1260 Infinity II LC system, equipped with a triple quadrupole MS (TQ MS), the Agilent 6470A triple quadrupole LC/MS. Method and kit suitability was verified by comparing linear correlation coefficients (R^2) and relative standard deviations (RSDs) with Agilent's requirements for OQ in positive-ion mode. Limit of detection (LOD) and limit of quantitation (LOQ) were 0.004 µg/mL and 0.011 µg/mL, respectively. Reproducible peak areas with RSDs of < 2.29% have been reported. An average R^2 of 0.99899887 ± 0.00034110 with RSD of 0.03% for the calibration curve has been reported. While the method presented is not to be used for performing OQ, the results verify that the LCMS OQPV Negative Mode Standard kit is a suitable calibration standard set solution for negative-ion mode OQ of MS systems.

Introduction

Linearity is a critical performance characteristic for quantitative LC/MS analysis, directly impacting the accuracy and reliability of reported results. In LC/MS systems, linearity is influenced by multiple factors, including ionization efficiency, sample matrix, detector response, acquisition parameters, and chromatographic reproducibility.[1,2] Therefore, OQ of an MS system is an essential component for its linearity verification, method development, and ongoing performance monitoring.

Research for oligonucleotide-based therapeutics and their ability to impact biological pathways has risen over recent years.[3-5] Generally, the analysis of oligonucleotides by LC/MS is carried out in negative-ion mode due to ionizability of phosphate groups in their structure.[6] Routine analyses for oligonucleotides are often performed in negative-ion mode using ion pairing reagents such as HFIP paired with TEA coupled with reverse-phase LC/MS.[6-8] Historically, the assessment of linearity has been performed in positive-ion mode, requiring the system to be carefully cleaned and flushed to remove any residual ion pairing reagents which could impact results.[5,9] Even though the qualification and calibration of instruments directly in negative-ion mode is now desired, the commercial market lacks suitable standards for this purpose.

In this study, we have developed an LC/MS method to demonstrate the utility of the Agilent LCMS OQPV Negative Mode Standard kit (p/n 5191-4567) for the assessment of detector and system linearity of an LC/MS system directly in negative-ion mode, using an Agilent 6470A triple quadrupole LC/MS as a model. This technical note highlights the kit's ability to eliminate the need for tedious and time-consuming instrument preparation required for performing OQ in positive ion-mode.

Methods and materials

Sample preparation

A calibration curve was prepared using calibration standards from the LCMS OQPV Negative Mode Standard kit (p/n 5191-4567; qty: 1 unit). The kit comprises six amber glass ampoules containing the calibration standard prepared at varying concentrations: 0.00 (reference blank), 0.25, 0.50, 1.00, 5.00, and 10.00 µg/mL. A volume containing 100 µL of each standard was transferred to individual corresponding Agilent autosampler vials (p/n 5182-0866; qty: 1 unit) for UHPLC/TQ MS analysis.

UHPLC/TQ MS analysis

Samples were separated and analyzed on an Agilent 1260 Infinity II LC system coupled to an Agilent 6470A triple quadrupole LC/MS comprising the following modules: (1) 1260 Infinity II Binary Pump (model G7112B); (2) 1260 Infinity II Multisampler (model G7167A); (3) 1260 Infinity II MCT (model G7116A); and (4) 6470A triple quadrupole LC/MS (model G6470A).

Instrument parameters and separation conditions for the 1260 Infinity II LC system are shown in Table 1.

Table 1. Agilent 1260 Infinity II LC system conditions.

Parameter	Value
Column	Agilent ZORBAX RR Eclipse Plus C18 column, 4.6 × 50 mm, 3.5 µm, 400 bar (p/n 959943-902)
Column Temperature	40 °C
Injection Volume	1.0 µL
Speed	Draw 100 µL/min; Eject 400 µL/min
Autosampler Temperature	10 °C
Needle Wash	Needle wash is 10 s with 33% methanol (v/v), 33% 2-propanol (v/v), and 33% Milli-Q water (v/v)
Mobile Phase	A) 5 mM ammonium formate in water B) 75% methanol in water (v/v) with 5 mM ammonium formate
Flow Rate	0.50 mL/min
Gradient Program	Time (min) %B 0.00 3.00 0.50 3.00 11.20 35.40 11.21 97.00 13.20 97.00 13.21 3.00 15.00 3.00
Stop Time	15 minutes
Post Run	0 minutes

The separation and analysis utilize a sample injection volume of 1.0 µL of each standard onto a ZORBAX RR Eclipse Plus C18 column (4.6 × 50 mm, 3.5 µm; p/n 959943-902) and separated using a linear gradient method at a constant flow rate of 0.5 mL/min and column temperature of 40 °C. The autosampler temperature was set to 10 °C. Mobile phase and needle wash solutions were prepared using the following reagents: Milli-Q water, InfinityLab Methanol for LC/MS (p/n 5191-5111-001) and 5M Ammonium Formate solution (p/n G1946-85021) obtained from Agilent, and 2-propanol (Sigma-Aldrich; sku 1027811000). The autosampler was set to perform a needle wash for 10 seconds with a solution of 33% methanol (v/v), 33% 2-propanol (v/v), and 33% Milli-Q water (v/v). The following 15-minute linear gradient was used: Mobile phase A: 5 mM ammonium formate in Milli-Q water (v/v) (unbuffered); Mobile phase B: 75% methanol in Milli-Q water (v/v), 5 mM ammonium formate (unbuffered). The following elution gradient was used: 0.00–0.50 min, 3.00% B; 0.51–11.20 min, 3.00–35.40% B; 11.21–13.20 min, 97.00% B; 13.21–15.00 min, 3.00% B.

Parameters for the Agilent Dual Jet Stream ESI source are shown in Table 2. The Dual Jet Stream ESI source was operated in negative-ion mode and used to introduce the sample into the 6470A triple quadrupole LC/MS. Capillary and nozzle voltages were set at –4,000 and –1,000 V, respectively. Nebulizer pressure was set to 17 psi. Drying and sheath gas flow rates were both set to 6 L/min. Nitrogen drying and sheath gas temperatures were both set to 200 °C.

Table 2. Agilent Dual Jet Stream ESI source parameters.

Parameter	Value
Ion Mode	Negative
Drying Gas Temperature	200 °C
Drying Gas Flow	6 L/min
Sheath Gas Temperature	200 °C
Sheath Gas Flow	6 L/min
Nebulizer Pressure	17 psi
Capillary Voltage	–4,000 V
Nozzle Voltage	–1,000 V

Scan and time parameters for the 6470A triple quadrupole LC/MS are listed in Table 3 and Table 4, respectively. The mass spectrometer was operated in selected ion monitoring (SIM) mode and in negative polarity. Samples from the LCMS OQPV Negative Mode Standard kit were monitored by setting the mass to *m/z* 545.1 with a dwell time of 470 ms and the MS2 resolution set to "Unit". Fragmentor and cell accelerator voltages were set to 140 and 5 V, respectively. Electron multiplier voltage (EMV) was adjusted to 1,435 V in manual tune mode. Acquired UHPLC/TQ MS data was retrieved using the Agilent MassHunter Workstation software (version 10.1, build 10.1.67) and analyzed with the Agilent MassHunter Quantitative Analysis software (version 10.2, build 10.2.733.8).

Table 3. Agilent 6470A triple quadrupole LC/MS scan segments and manual mode parameters.

Parameter	Value
Compound Group	"None"
Compound Name	LCMS OQPV Negative Mode Standard
ISTD	"Unchecked"
Mass	*m/z* 545.1
MS2 Res	Unit
Dwell	470 ms
Fragmentor	140 V
Cell Accelerator Voltage	5 V
Polarity	Negative
Electron Multiplier Voltage in Manual Tune Mode	1,425 V

Table 4. Agilent 6470A triple quadrupole LC/MS time segment parameters.

Parameter	Value
Number	1
Start	0
Scan Type	MS2 SIM
Div Valve	To MS
Delta EMV (+)	0
Delta EMV (–)	0
Stored	"Checked"

Results and discussion

The LCMS OQPV Negative Mode Standard kit was used to develop and demonstrate a quantitative method for the assessment of detector and system linearity based on an LC/MS system operated in negative-ion mode without the need for ion-pairing reagents or additional additives. The method comprises the separation and analysis of six calibration standards by LC/MS, followed by the evaluation and assessment of calibration curve linearity and quantitative reproducibility.

First, a 15-minute linear gradient based on reverse-phase C18 chromatography, along with a Dual Jet Stream ESI source and EMV parameters, was optimized and implemented to accurately and reproducibly quantitate the standards by their retention time and peak area. The LCMS OQPV Negative Mode Standard kit consists of a reference blank, along with five varying concentrations of calibration standard ranging from 0.25 to 10.00 µg/mL. The neutral monoisotopic mass of the standard is 546.1363 Da and its corresponding deprotonated mass, $[M-H]^-$ = *m/z* 545.1, was monitored using the 6470A triple quadrupole LC/MS operated in SIM mode and in negative polarity. To normalize the method across other instruments, the EMV (e.g., detector gain) was optimized so that the maximum instrument response for the highest concentration standard of 10.00 µg/mL was approximately 1.5×10^6. For the 6470A triple quadrupole LC/MS used in this study, the EMV was adjusted to 1435 V in manual tune mode. Nonetheless, the EMV or detector gain setting may differ depending on detector age and can vary from instrument to instrument.

LC/MS linearity and reproducibility were evaluated by triplicate injection analysis across five out of six calibration levels. The five calibration levels used to generate calibration curves consisted of calibration standards ranging in concentration from 0.25 to 10.00 µg/mL. Since the reference blank contains no measurable standard, it was not included as a calibration level nor was it used for linearity assessment. Representative total ion chromatograms of the six calibration standards are displayed in Figure 1. The calibration standards were observed to elute at a retention time of approximately 10.1 minutes. Signals ranging from a retention time window of approximately 12.5 to 15.0 minutes correspond to the "wash" segment of the LC separation and were therefore not included in the assessment of the method. Consistent retention times and symmetrical peak shapes were observed, indicating stable chromatographic performance throughout the analytical sequence. The MassHunter Quantitative Analysis software was used to generate a plot of the integrated total ion chromatogram peak area versus the nominal concentration of each calibration standard. A linear regression model was then applied by the software to assess calibration curve linearity for each of the three calibration curve replicates (Figure 2). The resulting 5-level average calibration curve demonstrates a strong linear relationship across the evaluated concentration range with an average R^2 of 0.99899887 ± 0.00034110 and an RSD of 0.03%. These results meet and exceed the R^2 acceptance criteria of > 0.98000 and RSD of < 10.00% for the operational qualification requirements for positive-ion mode defined by Agilent.[10]

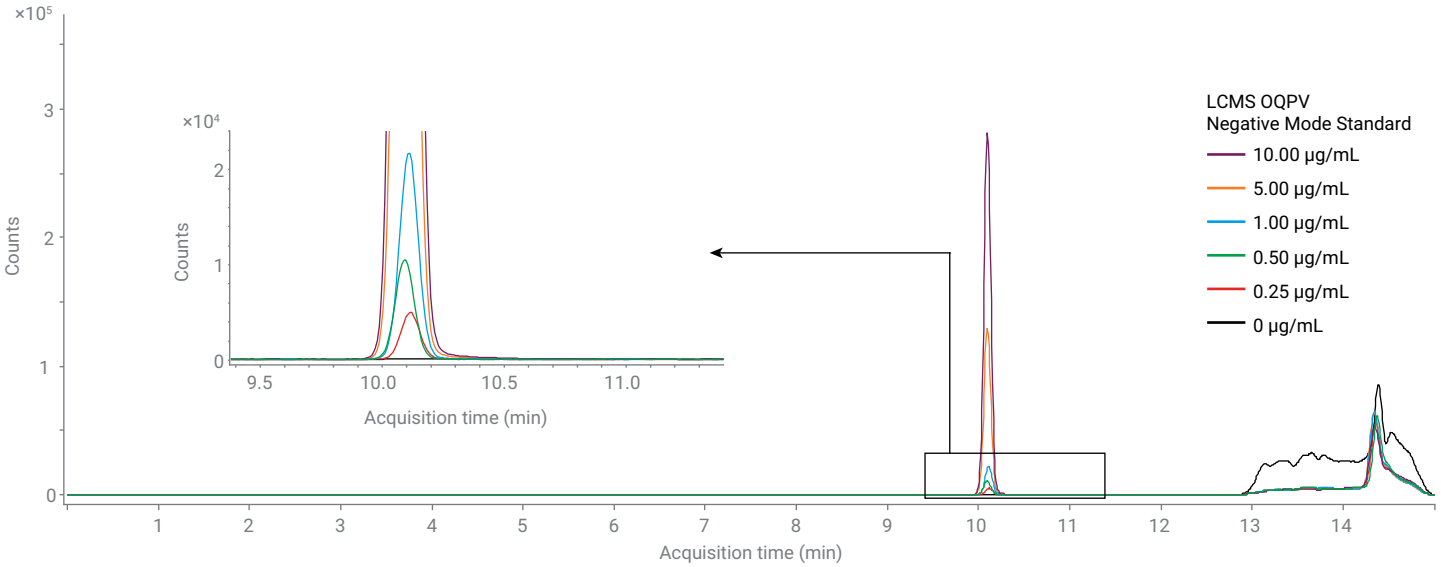

Figure 1. Representative total ion chromatograms for SIM $[M-H]^-$ = *m/z* 545.1 of all six calibration standards included in the Agilent LCMS OQPV Negative Mode Standard Kit (p/n 5191-4567) (n = 1 × 1.0 µL injection). Separation and analysis was performed on an Agilent ZORBAX RR Eclipse Plus C18 column, 4.6 × 50 mm, 3.5 µm, 400 bar (p/n 959943-902).

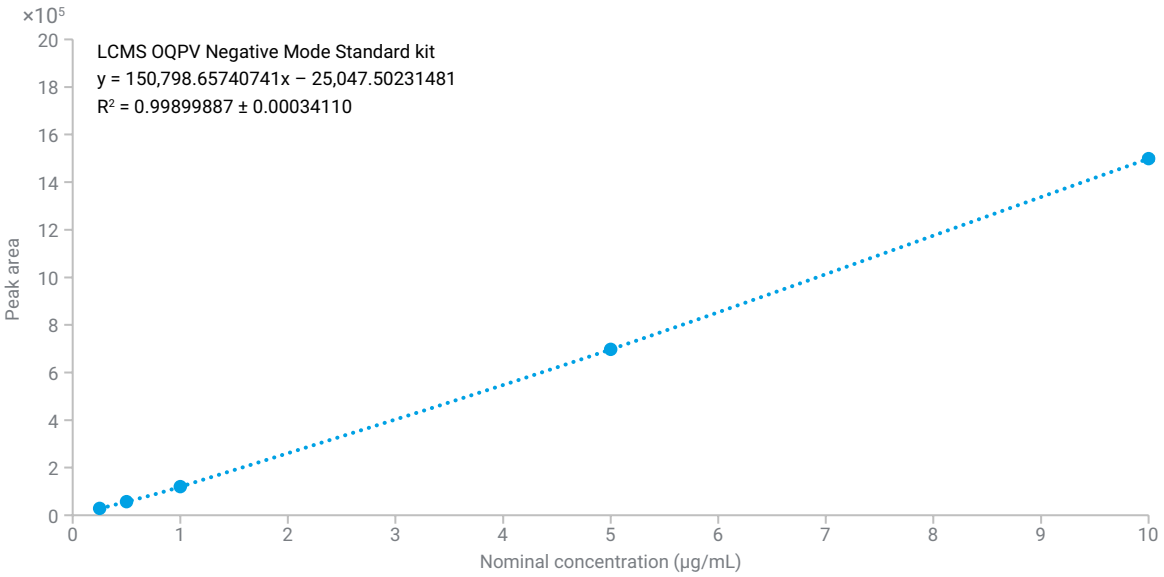

Figure 2. Average calibration curve for the Agilent LCMS OQPV Negative Mode Standard kit (p/n 5191-4567) (n = 3 × 1.0 µL injection replicates).

LOD, LOQ, and quantitative reproducibility of the LC/MS method were also determined and evaluated using all six calibration standards included in the kit. The LOD was calculated using the following equation: , where is the standard deviation of the blank, and is the slope of the calibration curve. The LOQ was then calculated using Equation 1.

Equation 1.

$$LOD = 3.3 \frac{\sigma}{S}$$

Where σ is the standard deviation of the blank, and S is the slope of the calibration curve.

The LOQ was then calculated using Equation 2.

Equation 2.

$$LOQ = 10 \frac{\sigma}{S}$$

The LOD and LOQ were calculated to be 0.004 µg/mL and 0.011 µg/mL, respectively. Peak areas, standard deviations, RSDs, and R² values are shown in Table 5. RSDs of < 2.29% were determined for peak areas of the standards demonstrating exceptional quantitative reproducibility for optimized LC/MS method when compared to the required RSD of < 10.00% for the operational qualification requirements for positive-ion mode as described by Agilent.[10]

Table 5. Peak areas, standard deviations, RSDs, and R² values for the Agilent LCMS OQPV Negative Mode Standard kit (p/n 5191-4567) (n = 3 × 1.0 µL injection replicates).

Nominal Concentration (µg/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	Standard Deviation	%RSD
0.00 (Reference Blank)	428	687	728	614	163	26.48
0.25	27528	28675	28626	28276	649	2.29
0.50	56469	56792	57525	56929	541	0.95
1.00	119956	118212	120786	119651	1314	1.10
5.00	701995	694611	694281	696962	4362	0.63
10.00	1488795	1501505	1506164	1498821	8990	0.60
Linear Correlation Coefficient (R^2)	0.99936599	0.99883072	0.99873206	0.99897626	0.00034110	0.03

Conclusion

In this study, an LC/MS method was developed to evaluate the Agilent LCMS OQPV Negative Mode Standard kit (p/n 5191-4567) as a suitable calibration standard set for use in operational qualification of mass spectrometry instruments in negative-ion mode. The method features an optimized 15-minute linear gradient based on reverse-phase C18 chromatography, along with an optimized Agilent Dual Jet Stream ESI source and EMV parameters demonstrated on an Agilent 1260 Infinity II LC system coupled to an Agilent 6470A triple quadrupole LC/MS. The suitability of the method and kit was validated by comparing determined linear correlation coefficients and relative standard deviations to the acceptance criteria set by Agilent for operational qualification requirements for positive-ion mode. While the method developed is not to be used for performing OQ, the results verify that the Agilent LCMS OQPV Negative Mode Standard kit is a suitable calibration standard set solution which will allow for OQ of mass spectrometers to be performed exclusively in negative-ion mode. The kit offers a significant advantage for users who prefer to eliminate the need for tedious cleansing of ion-pair reagents from the MS system in preparation for operational qualification in positive-ion mode, a pain point for users with dedicated instruments routinely operating in negative-ion mode for oligonucleotide analyses.

References

1. Cheng, W. L.; Markus, C.; Lim, C. Y.; Tan, R. Z.; Sethi, S. K.; Loh, T. P. Calibration Practices in Clinical Mass Spectrometry: Review and Recommendations. *Ann. Lab. Med*. **2023**, *43(1)*, 5–18. DOI: 10.3343/alm.2023.43.1.5
2. Cortese, M.; Gigliobianco, M. R.; Magnoni, F.; Censi, R.; Di Martino, P. Compensate for or Minimize Matrix Effects? Strategies for Overcoming Matrix Effects in Liquid Chromatography–Mass Spectrometry Technique: A Tutorial Review. *Molecules* **2020**, *25*, 3047. DOI: 10.3390/molecules25133047
3. Thakur, S.; Sinhari, A.; Jain, P.; Jadhav, H. R. A Perspective on Oligonucleotide Therapy: Approaches to Patient Customization. *Front. Pharmacol*. **2022**, *13*, 1006304. DOI: 10.3389/fphar.2022.1006304
4. Takakusa, H.; Iwazaki, N.; Nishikawa, M.; Yoshida, T.; Obika, S.; Inoue, T. Drug Metabolism and Pharmacokinetics of Antisense Oligonucleotide Therapeutics: Typical Profiles, Evaluation Approaches, and Points to Consider Compared with Small Molecule Drugs. *Nucleic Acid Ther*. **2023**, *33(2)*, 83–94. DOI: 10.1089/nat.2022.0054
5. Liu, A.; Cheng, M.; Zhou, Y.; Deng, P. Bioanalysis of Oligonucleotide by LC–MS: Effects of Ion-Pairing Reagents and Recent Advances in Ion-Pairing-Free Analytical Strategies. *Int. J. Mol. Sci.* **2022**, *23*, 15474. DOI: 10.3390/ijms232415474
6. Guzmán-Lorite, M.; Marina, M. L.; García, M. C.; Gabelica, V. miRNA and DNA Analysis by Negative Ion Electron Transfer Dissociation and Infrared Multiple-Photon Dissociation Mass Spectrometry. *Anal. Chim. Acta* **2024**, *1299*, 342431. DOI: 10.1016/j.aca.2024.342431
7. Apffel, A.; Chakel, J. A.; Fischer, S.; Lichtenwalter, K.; Hancock, W. S. Analysis of Oligonucleotides by HPLC–Electrospray Ionization Mass Spectrometry. *Anal. Chem.* **1997**, *69*, 1320–1325. DOI: 10.1021/ac960916h
8. Sutton, J.; Guimaraes, G. J.; Annavarapu, V.; Dongen, W. D.; Bartlett, M. G. Current State of Oligonucleotide Characterization Using Liquid Chromatography–Mass Spectrometry: Insight into Critical Issues. *J. Am. Soc. Mass Spectrom*. **2020**, *31*, 1775–1782. DOI: 10.1021/jasms.0c00179
9. Gong, L. Comparing Ion-Pairing Reagents and Counter Anions for Ion-Pair Reversed-Phase Liquid Chromatography/Electrospray Ionization Mass Spectrometry Analysis of Synthetic Oligonucleotides. Rapid Commun. *Mass Spectrom*. **2015**, *29(24)*, 2402–2410. DOI: 10.1002/rcm.7409
10. Analytical Scale Agilent LCMS Systems Operational Qualification. Agilent Technologies, December 2018. Available at: https://www.agilent.com/Library/service/Public/LCMS%20Hardware_Operational%20Qual-LC_col7.pdf

www.agilent.com

DE-014468

Printed in the USA, May 1, 2026
5994-9136EN

Agilent
Trusted Answers