

Pharma and biopharma

LC-UV analysis of large RNA using an inert UHPLC system

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Keywords

Oligonucleotide analysis,
Vanquish Amplify UHPLC system,
large RNA, mRNA, SurePac
Oligo RP MDi column

Test benefits

- Increased sensitivity of metal-sensitive analytes such as adenosine 5'-(α,β -methylene) diphosphate (AMPcP).
- Consistent, reproducible peak area precision, even for samples with low analyte concentrations.
- Accurate results with the first sample injection eliminating the need for conditioning, thus saving time and sample.

Goal

Evaluate the performance of the Thermo Scientific™ Vanquish™ Amplify UHPLC System for analysis of metal-sensitive analytes such as AMPcP and large RNA.

Introduction

The analysis of oligonucleotide-based therapeutics by LC–UV and LC–MS/MS demands methods that deliver high sensitivity, accuracy, and reproducibility. Reliable quantification is particularly challenging at low concentrations due to the inherent physicochemical properties of these analytes, making robust and consistent analytical performance critical.

Oligonucleotides possess a negatively charged phosphodiester backbone ($pK_a \sim 2$), with a phosphodiester oxygen on each nucleotide contributing one negative charge, except at the 5-terminus, where there are two. These groups form strong bonds with metals, particularly with “hard” metal ions such as Na^+ , K^+ , Mg^{2+} , Fe^{3+} , and Cr^{3+} .¹ In conventional systems containing stainless-steel components, these interactions lead to analyte adsorption within the sample flow path, causing peak broadening, reduced peak area, and compromised quantitative accuracy.

Large RNA is similar in physicochemical behavior to mRNA but lacks the 7-methylguanosine as 5'-cap and a poly(A) tail. Large RNA standards are widely used as surrogates in method development for mRNA separations.² Both large RNA and

mRNA present significant analytical challenges due to their size and susceptibility to metal interactions. While countermeasures such as sample flow path conditioning by analysis in phosphate buffer / acids or the injection of sacrificial (“throwaway”) oligonucleotides can reduce these effects,³ they may add complexity to the workflow.

Replacing stainless-steel components with biocompatible (corrosion-resistant) materials—such as capillaries, needles, flow cells, and injection valves—significantly improves analytical performance. The Thermo Scientific™ Vanquish™ Flex and Thermo Scientific™ Vanquish™ Horizon UHPLC Systems, which utilize MP35N fluidic components, are therefore classified as biocompatible systems. MP35N is a corrosion-resistant, iron-free alloy widely used in medical implants that effectively minimizes metal–analyte interactions. These interactions can be further reduced through permanent modification of flow path surfaces using metal-free, low-adsorption (inert) coatings.

Building on this approach, the Thermo Scientific™ Vanquish™ Amplify UHPLC Platform incorporates a fully inert sample flow path from the injection valve to the detector, effectively minimizing analyte interaction with metal surfaces. In this study, the performance of the Vanquish Amplify UHPLC system was evaluated for the analysis of metal-sensitive analytes, including AMPcP and large RNA molecules (500 and 5,000 nucleotides) as summarized in Figure 1.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification (Cat No. 50136149)
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (Cat No. A955)
- Applied Biosystems™ Triethylammonium acetate (TEAA), 2.0 M (Cat No. 400613)
- Invitrogen™ Millennium™ RNA Markers (Mix of 10 large RNAs. The 500 nt and 5,000 nt RNAs were ordered separately as a custom format) (Cat No. AM7150)
- Sigma-Aldrich Adenosine 5′-(α,β-methylene) diphosphate (Cat No. M3763)
- Thermo Scientific™ Chemicals Adenosine (Cat No. 164040050)
- Fisher Scientific™ Trifluoroacetic acid, Optima™ LC/MS grade (Cat No. A116)
- Fisher Scientific™ Isopropanol, Optima™ LC/MS grade (Cat No. A461)
- Fisher Scientific™ Methanol, Optima™ LC/MS grade (Cat No. A456)

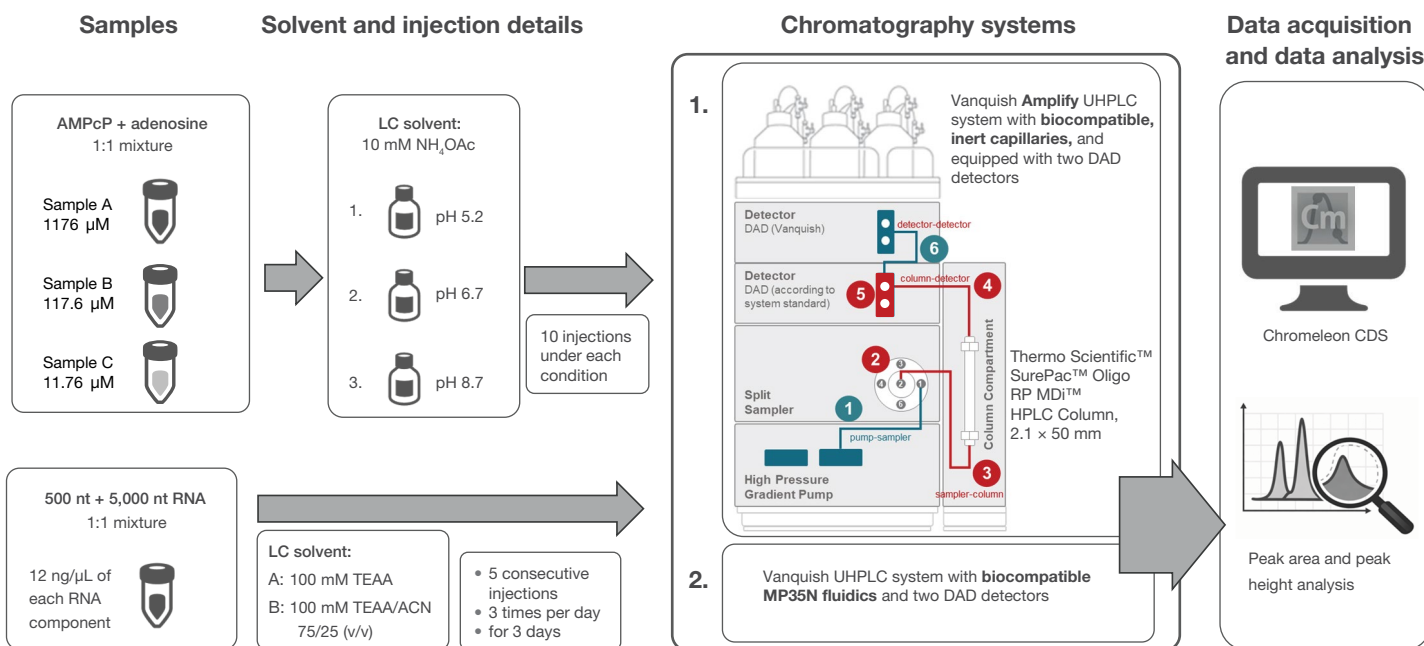


Figure 1. Overview of the performance assessment study of the Vanquish Amplify UHPLC system. The system parts labeled in blue versus red include (1) pump-to-autosampler capillary (Thermo Scientific™ Viper™ TQ Capillary, 0.1 × 350 mm, MP35N); (2) autosampler needle, needle seat and 25 μ L sample loop (all inert); (3) sample-to-column capillary (Viper TQ, active preheater, 0.1 × 350 mm, inert); (4) column-to-detector capillary (Viper TQ capillary, 0.1 × 380 mm, inert); (5) semi-micro flow cell (inert), and (6) detector to detector capillary (0.18 × 650 mm, MP35N).

- Fisher Scientific™ Ammonium acetate, Optima™ LC/MS grade (Cat No. A114)
- Fisher Scientific™ Ammonium hydroxide, Optima™ LC/MS grade (Cat No. A470)
- Thermo Scientific™ Chemicals Acetic acid, 99.8 %, for analysis (Cat No. 222140010)
- Thermo Scientific™ Vanquish™ Semi-Micro Flow Cell, Inert, 2.5 µL, 7 mm (*used in first DAD*) (Cat No. 6083.0580)
- Thermo Scientific™ Vanquish™ Semi-Micro Biocompatible Flow Cell, MP35N, 7 mm, 2.5 µL (*used in 2nd DAD*) (Cat No. 6083.0550)

Sample handling

- Thermo Scientific™ Finnpiquette™ F2 Variable Volume Single-Channel Pipette: 100–1,000 µL (Cat No. 4642090)
- Thermo Scientific™ Finnpiquette™ F2 Variable Volume Single-Channel Pipette: 10–100 µL (Cat No. 4642070)
- Thermo Scientific™ Finnpiquette™ F2 Variable Volume Single-Channel Pipette: 1–10 µL (Cat No. 4642030)
- Fisherbrand™ Mini Vortexer (Cat No. 14-955-152)
- Thermo Scientific™ Orion™ 3-Star pH Benchtop Meter (Cat No. 1112106)
- Thermo Scientific™ SureSTART™ 2 mL Amber Glass Short Thread Screw Top Vials, 100/pack, Level 2 (Cat No. 6ASV9-2P)
- Thermo Scientific™ SureSTART™ Blue Polypropylene 9 mm AVCS™ Screw Caps with Soft Blue Silicone/Clear PTFE Septa, 100/pack, Level 3 (Cat. No. 6PSC9ST101)

Instrumentation

1) Vanquish Amplify system

Vanquish Flex Amplify and Vanquish Horizon Amplify UHPLC systems were both used. The Vanquish Flex Amplify UHPLC system was used for the large RNA test, and the Vanquish Horizon Amplify UHPLC was used for the small molecule test.

- Vanquish Amplify UHPLC system consisting of:
 - Thermo Scientific™ System Base Vanquish Amplify (Cat No. VF-S03-A-03)
 - Thermo Scientific™ Vanquish™ Binary Pump F/H (Cat No. VF-P10-A-01/VH-P10-A-02)
 - Thermo Scientific™ Vanquish™ Split Sampler FT/HT Amplify (Cat No. VF-A14-A-03/VH-A14-A-02)
 - Thermo Scientific™ Vanquish™ Column Compartment H (Cat No. VH-C10-A-03)
 - Two (2) Thermo Scientific™ Vanquish™ Diode Array Detectors FG (Cat No. VF-D11-A-01)

2) Vanquish UHPLC system with MP35N capillaries

- Vanquish UHPLC system consisting of:
 - Thermo Scientific™ System Base Vanquish™ Horizon/Flex (Cat No. VF-S01-A-03)
 - Thermo Scientific™ Vanquish™ Binary Pump F/H (Cat No. VF-P10-A-01/VH-P10-A-02)
 - Thermo Scientific™ Vanquish™ Split Sampler FT/HT (Cat No. VF-A10-A-03/VH-A10-A-02)
 - Thermo Scientific™ Vanquish™ Column Compartment H (Cat No. VH-C10-A-03)
 - Two (2) Thermo Scientific™ Vanquish™ Diode Array Detectors FG (Cat No. VF-D11-A-01)
 - Thermo Scientific™ Vanquish™ Semi-Micro Biocompatible Flow Cell, MP35N, 7 mm, 2.5 µL (*used in first DAD*) (Cat No. 6083.0550)
 - Thermo Scientific™ Vanquish™ Semi-Micro Flow Cell, SST, 7 mm, 2.5 µL (*used in 2nd DAD*) (Cat No. 6083.0530)

3) Waters Acquity Premier UPLC system

Waters™ ACQUITY™ Premier UPLC™ system with MaxPeak™ High Performance Surfaces (HPS) Technology (Waters Corp.) consisting of binary pump, autosampler, column heater, photodiode array detector, and flow cell in standard configuration. Identical Vanquish DADs were added as second detectors on all systems after the standard UV detectors to provide comparable data.

- Thermo Scientific™ Vanquish™ Diode Array Detector FG (Cat No. VF-D11-A-01)
- Thermo Scientific™ Vanquish™ Semi-Micro Biocompatible Flow Cell, MP35N, 7 mm, 2.5 µL (Cat No. 6083.0550)

Sample preparation

AMPcP/adenosine sample

AMPcP and adenosine were weighed and co-dissolved together in water to generate an equimolar solution with a concentration of 1,176 µM per compound (sample A). This solution was serially diluted 10-fold with water to 117.6 µM (sample B) and 11.76 µM (sample C).

Large RNA sample

Two large RNA components (500 nt and 5,000 nt), also present in the commercially available Millennium RNA Ladder, were obtained at a concentration of 1 mg/mL. Each RNA sample was diluted separately in RNase-free water and then combined in a 1:1 ratio to yield a final concentration of 12 ng/ μ L per component. The sample was aliquoted and stored at $-80\text{ }^{\circ}\text{C}$, and a fresh aliquot was thawed for daily use. Analyses were performed using a 1 μ L injection volume, corresponding to 12 ng per RNA component and a total sample amount of 24 ng loaded on column.

Mobile phase preparation

The 50 mM wash and 10 mM mobile phase ammonium acetate solutions were prepared by dissolving ammonium acetate in ultrapure water, followed by pH adjustment and filtration through a 0.45 μ m filter.

The 100 mM TEAA was prepared from a commercially available concentrate of 2.0 M triethylammonium acetate by dilution in ultrapure water. Eluent A was prepared by measuring the required amount of concentrate in a graduated cylinder and adding it to water from the ultrapure water system dispenser. Eluent B for the large RNA test was mixed by adding 98.25 g (125 mL) acetonitrile to 375 g of eluent A. Commercially available concentrate was used because the preparation from strong acid and strong base requires subsequent pH correction and filtering, which can introduce contamination and require additional analyst time. The mobile phases were freshly prepared every three days.

Chromatographic conditions

To enable a direct comparison of flow path materials, the inert Vanquish Amplify UHPLC system was compared to a Vanquish UHPLC system equipped with biocompatible MP35N fluidics.

The Vanquish Amplify system differs from the Vanquish MP35N system in several fluidic components, including the inert components needle, needle seat, 25 μ L sample loop, 0.1 \times 380 mm active preheater TQ, the 0.1 \times 350 mm column-to-detector Viper TQ capillary, and the flow cell, while all other system components and operating conditions were kept identical for comparison. The fluidics outside of the sample flow path were not different. SurePac Oligo RP MDi columns with inert hardware and a spherical monodisperse supermacroporous polymer resin stationary phase were used for all tests to ensure comparability of results. Columns were chosen to be as similar as possible by reviewing quality control data.

Table 1. Chromatographic conditions used for the AMPcP /adenosine sample.

Parameter	Value
Column	Thermo Scientific™ SurePac™ Oligo RP MDi™ Column, 2.5 μ m, 2.1 \times 50 mm (Cat. No: 43712-052132)
Solvent A	10 mM ammonium acetate (NH_4OAc) with pH adjusted to: 5.2 / 6.7 / 8.7
Solvent B	N/A
Rear seal wash	75% isopropanol with 0.1% formic acid
Method length	Isocratic - 2 min
Flow rate	200 μ L/min
Column temperature	30 $^{\circ}\text{C}$, still air mode, active preheater 30 $^{\circ}\text{C}$
Autosampler temperature	5 $^{\circ}\text{C}$
Needle wash mode	No wash
Injection volume	1 μ L
Sample	Three concentration levels of an equimolar AMPcP/adenosine mixture Sample A: 1176 μ M Sample B: 117.6 μ M Sample C: 11.76 μ M
Detector settings	260 nm, 20 Hz, 0.200 s response time

Table 2. Chromatographic conditions used for large RNA mixed sample.

Parameter	Value																
Column	SurePac Oligo RP MDi HPLC column, 2.5 μ m 2.1 \times 50 mm (Cat. No. 43712-052132)																
Solvent A	100 mM triethylammonium acetate (TEAA)																
Solvent B	100 mM TEAA/acetonitrile (ACN) 75/25 (v/v)																
Rear seal wash	75% isopropanol with 0.1% formic acid																
Gradient	<table border="1"><thead><tr><th>Time [min]</th><th>B [%]</th></tr></thead><tbody><tr><td>-1</td><td>30</td></tr><tr><td>0</td><td>30</td></tr><tr><td>3.3</td><td>65</td></tr><tr><td>3.3</td><td>90</td></tr><tr><td>4.5</td><td>90</td></tr><tr><td>4.5</td><td>30</td></tr><tr><td>9.0</td><td>30</td></tr></tbody></table>	Time [min]	B [%]	-1	30	0	30	3.3	65	3.3	90	4.5	90	4.5	30	9.0	30
Time [min]	B [%]																
-1	30																
0	30																
3.3	65																
3.3	90																
4.5	90																
4.5	30																
9.0	30																
Flow rate	400 μ L/min																
Column temperature	50 $^{\circ}\text{C}$, still air mode, active preheater 50 $^{\circ}\text{C}$																
Autosampler temperature	5 $^{\circ}\text{C}$																
Needle wash mode	No wash																
Injection volume	1 μ L, or 2 μ L for injected wash solution																
Detector 1 and 2 settings	260 nm, 20 Hz, 0.200 s response time																

AMPcP/adenosine analysis

The Vanquish Horizon system with DAD FG and equivalent Amplify system were evaluated using three concentration levels of an equimolar AMPcP/adenosine mixture (samples A–C). Samples were analyzed in order of increasing concentration, starting from the lowest. Each concentration was analyzed at three different pH values (pH 5.2, 6.7, and 8.7) and injected ten times to assess reproducibility. Chromatographic conditions are provided in Table 1.

Large RNA analysis

For large RNA analysis, RNA of 500 and 5,000 nucleotides in length were analyzed using an ion-pair reversed-phase method on a SurePac Oligo RP MDi column featuring inert column hardware (Table 2).

The injection sequence shown in Table 3 was repeated three times per day over three consecutive days (15 injections of RNA sample per day, 45 injections in total).

Table 3. Injection list for one set of injections, repeated three times daily for three consecutive days.

Injection #	Injection name
1	Blank
2	Blank
3	Blank
4	Inject water
5	Large RNA Inj. 1
6	Large RNA Inj. 2
7	Large RNA Inj. 3
8	Large RNA Inj. 4
9	Large RNA Inj. 5
10	Blank
11	Wash with 0.1% TFA in organic

The wash with 0.1% trifluoroacetic acid (TFA) was performed using a 20-minute method, including a 2 μ L injection of the wash solution. During this step, the system was flushed with a mixture of 25/25/25/25 (v/v) water, isopropanol, acetonitrile, and methanol containing 0.1% TFA. Systems were set in standby at the end of each day using 80/20 acetonitrile/water at 400 μ L/min for 20 minutes, followed by an overnight flow of 50 μ L/min for approximately 16 hours.

In addition, the Vanquish Amplify UHPLC system was compared to a third-party UPLC system featuring MaxPeak High Performance Surfaces (HPS) technology fluidics, using identical DAD detectors that were added after the system UV flow cell to provide comparable data for the two systems (Figure 1).

Chromatography Data System

The Thermo Scientific™ Chromeleon™ 7.3.2 MUc Chromatography Data System (CDS) was used for all data acquisition and analysis.

Results and discussion

Evaluation of metal interactions using a probe analyte: MP35N versus inert fluidics

To probe metal–analyte interactions, AMPcP was analyzed in combination with adenosine, a non-metal-binding reference compound. As a highly polar, multivalent anion, AMPcP binds well to metallic surfaces, whereas adenosine does not. The AMPcP-to-adenosine peak ratio therefore serves as a reliable indicator of analyte loss due to surface interactions.

A 10 mM ammonium acetate mobile phase was selected due to its dual buffering capacity (pH 3.75–5.75 and 8.2–10.2). Three pH conditions were evaluated: pH 5.2 (adjusted with acetic acid), pH 6.7 (unadjusted), and pH 8.7 (adjusted with ammonium hydroxide). This range enables assessment of secondary surface interactions under different pH conditions (Figure 2).

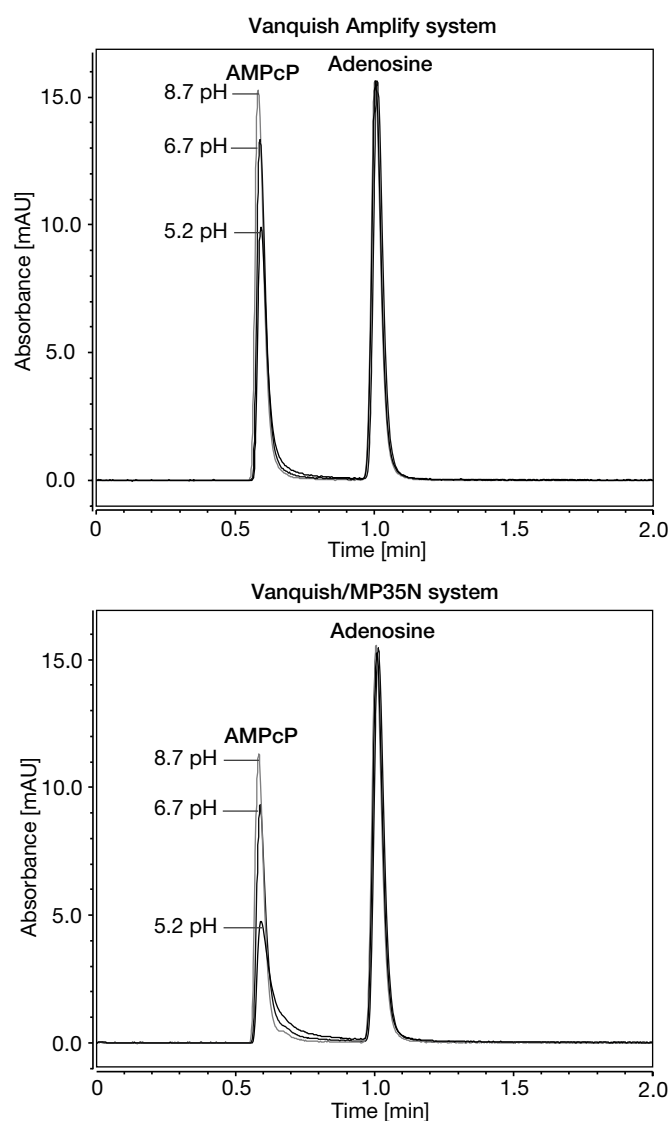


Figure 2. Chromatographic profiles obtained from the lowest concentration (11.76 μ M) sample of AMPcP/adenosine under three different mobile phase pH conditions, acquired on the Vanquish Amplify system (top) and the Vanquish/MP35N system (bottom).

These results demonstrate a clear dependence of secondary surface interactions on both pH and analyte concentration (Figure 3). Acidic conditions significantly enhance nonspecific surface interactions, particularly at lower analyte concentrations. Under the most challenging conditions (pH 5.2 and 11.76 μM), the inert sample flow path provided approximately a twofold increase in sensitivity compared to the conventional MP35N system. As analyte concentration increased and more basic mobile phase conditions were applied, the relative benefit of the inert sample flow path diminished, indicating reduced impact of surface interactions under these conditions.

Overall, these findings highlight the importance of flow path inertness for the accurate analysis of highly polar, anionic compounds such as AMPcP, particularly at low concentrations and under acidic mobile phase conditions. These findings provide a mechanistic basis for the improved performance observed for large RNA under comparable conditions.

Large RNA, comparison of MP35N and inert fluidics

The results obtained with AMPcP demonstrate the impact of metal-analyte interactions on chromatographic performance. Based on this mechanistic understanding, the performance of the Vanquish Amplify UHPLC system was further evaluated using large RNA as a representative analyte.

The Vanquish Amplify system consistently delivers outstanding results for analytes, whether metal-coordinating or not, with or without washing. For purposes of comparison, however, systems including columns and flow cells were subjected,

unless otherwise specified, to a 20-minute method in which the system was flushed with 0.1% TFA in organic as specified in the Experimental section. The purpose of the wash was to quickly remove any RNA carryover from the column stationary phase. The analysis was then carried out as described in Table 2.

MP35N and Vanquish Amplify system fluidics could not be compared using the TFA wash because the wash made the performance of both fluidics identical. Similar results have been obtained with 0.1% formic acid washes. For this reason, new MP35N fluidics were used for the comparison and a wash of 50 mM ammonium acetate at pH 9.2 in water was implemented. High grade, low metal content reagents were used to prevent metal ions from additives or solvents lodging in the column stationary phase and affecting the results.

The benefits of the Vanquish Amplify UHPLC system with the SurePac Oligo RP MDi column relative to an MP35N system with the same column are shown in Figure 4, Figure 5, and Table 4. An improvement of 1.35-fold for the shorter oligonucleotide and 1.25-fold for the longer oligonucleotide on the first injection is shown. Some saturation behavior was also observed with the MP35N fluidics. Specifically, starting with the third injection, peak areas were constant. Three injections are therefore required to obtain a stable peak area. These results highlight the improved performance of the Vanquish Amplify UHPLC system under these conditions, delivering enhanced sensitivity, lower relative standard deviation for peak area and height, and more reliable performance for demanding oligonucleotide analyses.

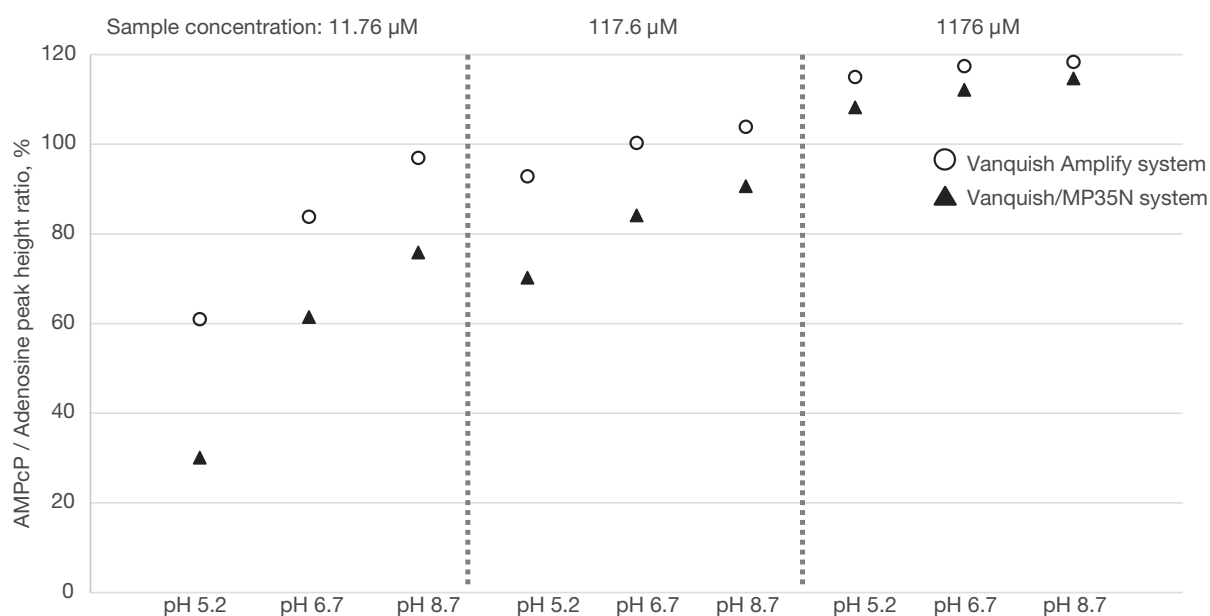


Figure 3. Peak height ratio of AMPcP and adenosine across three concentrations and three mobile phase pH conditions. Each point is the average of ten injections. Data acquired on the Vanquish Amplify system are represented by white circles and the Vanquish system with MP35N as black triangles.

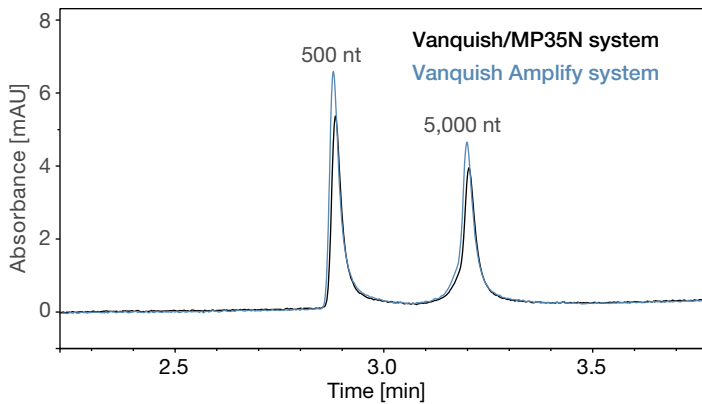


Figure 4. UV chromatograms of the first injection of 500 nt and 5,000 nt RNA sample on the Vanquish Amplify UHPLC system (blue) compared to the Vanquish UHPLC system with MP35N fluidics (black), demonstrating higher peak heights and areas for the Vanquish Amplify system.

Table 4. Representative improvement in peak area for the first injection on Vanquish Amplify UHPLC system compared to the Vanquish UHPLC system with MP35N fluidics.

System	500 nt (area, mAU*min)	500 nt (height, mAU)	5,000 nt (area, mAU*min)	5,000 nt (height, mAU)
Vanquish Amplify	0.23	6.48	0.20	4.45
Vanquish/MP35N	0.17	5.22	0.16	3.73
Improvement factor of Vanquish Amplify system over Vanquish/MP35N system	1.35x	1.24x	1.25x	1.19x

Large RNA, cross-vendor comparison of systems

The performance of the Vanquish Amplify UHPLC system and the Acquity Premier UPLC system for large RNA analysis was evaluated by comparing peak areas and heights of the 500 nt and 5,000 nt RNA. Each system was equipped with a matched SurePac Oligo RP MDi column to ensure comparable chromatographic conditions. Results were obtained using identical DAD UV detectors installed downstream of the system UV detector to ensure consistent detection conditions.

As shown in Figure 6 and Table 5, both systems produced comparable peak areas and heights for the fifth injection in a series of five injections, demonstrating a high level of system-to-system reproducibility. Such consistency is important for ensuring data comparability across instruments, minimizing variability during method transfer, and increasing confidence in the reliability of quantitative results.

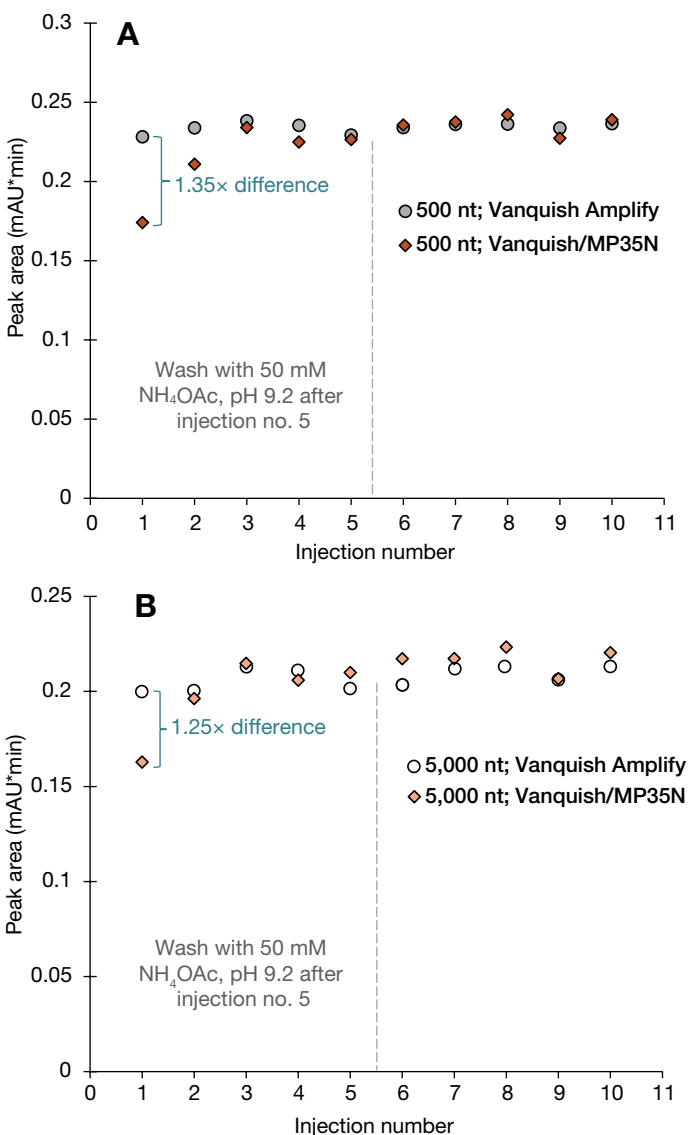


Figure 5. Comparison of peak areas obtained for 500 nt and 5,000 nt RNA on the Vanquish Amplify and Vanquish/MP35N systems, demonstrating improvement factors of 1.35 and 1.25, respectively, achieved with the inert fluidics of the Vanquish Amplify system.

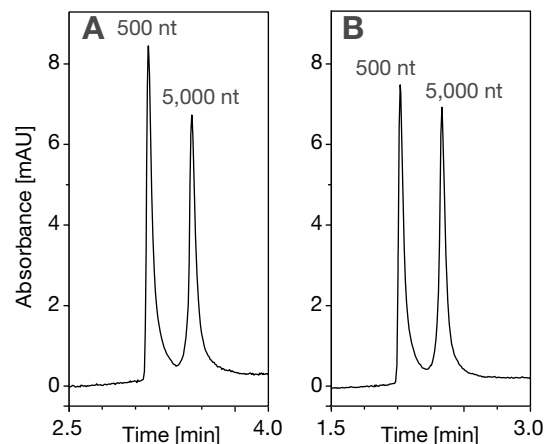


Figure 6. Comparison of UV chromatograms acquired on the second detector of the Vanquish Amplify system (A) and (B) the Acquity Premier system (B), each obtained from RNA sample injection no. 5.

Table 5. Peak areas and heights for the chromatograms in Figure 6.

System	500 nt (area, mAU*min)	500 nt (height, mAU)	5,000 nt (area, mAU*min)	5,000 nt (height, mAU)
Vanquish Amplify	0.51	8.28	0.48	6.50
Acquity Premier	0.42	7.40	0.42	6.79

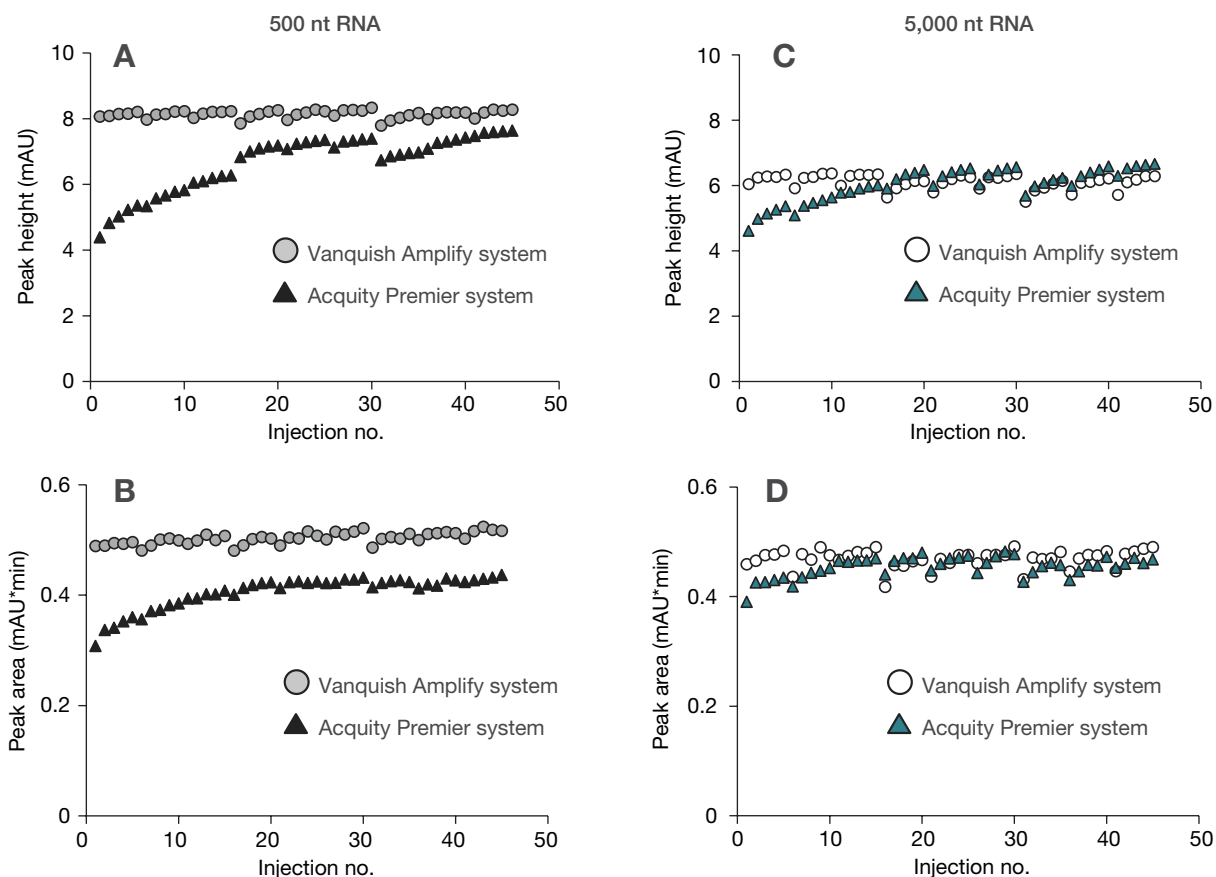


Figure 7. Peak height (A, C) and peak area (B, D) for all injections from the system comparison experiment for the 500 nt peak (A, B) and the 5,000 nt peak (C, D). The sample was injected five times, followed by a TFA wash; this sequence was repeated three times per day over three days.

Across the full injection series (Figure 7), similar trends were observed for the 5,000 nt RNA on both systems. For the 500 nt RNA, the Acquity Premier system exhibited saturation behavior, with increasing peak area over successive injections. This behavior, also observed to a lesser extent in later injections for both systems, is likely due to complex interactions between the analyte, ion-pairing reagents, and the stationary phase rather than the wetted flow path alone.

Conclusion

In this study, we have evaluated the system inertness of the Vanquish Amplify UHPLC system using AMPcP/adenosine and large RNA samples with a particular focus on mitigating metal-analyte interactions and their impact on sensitivity. The data obtained show:

- Using AMPcP as a probe analyte, the system provided up to a twofold increase in sensitivity compared to systems with MP35N fluidics under conditions where metal interactions are most pronounced, particularly at low concentrations and under acidic mobile phase conditions.

- For large RNA, the Vanquish Amplify UHPLC system delivered 1.25–1.35x higher signal intensity for the first injection compared to systems with MP35N fluidics, enabling reduced sample consumption and improved quantitative reliability. In addition, stable results were obtained from the first injection, eliminating the need for system conditioning.
- The Vanquish Amplify UHPLC system demonstrated comparable system inertness under the tested conditions for larger RNA fragments (5,000 nt) and slightly better system inertness for smaller fragments (500 nt) in this study than the MaxPeak HPS containing system, based on peak height and area comparisons using an external UV detector.
- The results demonstrate that the Vanquish Amplify UHPLC system significantly reduces metal-analyte interactions through its fully inert sample flow path, resulting in improved chromatographic performance and consistent, reproducible peak area precision for challenging samples and analytes at low concentrations.

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TN004606-EN 0526S