Application Note

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# Quantitative Bioanalysis of Oligonucleotides Using a 6495D Triple Quadrupole LC/MS System Combined with an Automated SPE Workstation


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## Abstract

This study demonstrates the significant advantages of using an automated solid phase extraction (SPE) system based on the Agilent Bravo liquid handling platform, in combination with the Agilent 6495D triple quadrupole LC/MS system, for oligonucleotide bioanalysis. These systems provide a complete solution from sample preparation to data analysis. Using the quantification of mipomersen[1] (trade name Kynamro) in pig plasma, as an example, we showcase the excellent performance of the 6495D triple quadrupole LC/MS system with high sensitivity and low detection limits from a complex matrix. Additionally, the automated SPE workstation reduces manual intervention time and improves operational consistency and reliability. The results indicate that this integrated automated solution could replace manual operations when conducting bioanalytical studies for oligonucleotide samples in a high-throughput environment, without compromising superior sensitivity, accuracy, and reproducibility.

## Introduction

In drug development, the quantitative analysis of oligonucleotides in biological samples is crucial for pharmacokinetics (PK), pharmacodynamics (PD), and toxicological safety evaluations. Due to the associated structural complexity of oligonucleotides and the unique nature of biological matrices, the corresponding sample preparation techniques are often complex. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are commonly used sample preparation methods, with SPE being more suitable to meet lower limit of quantitation (LLOQ) requirements and complex matrices.[2] However, traditional manual SPE methods are time consuming and prone to human error.

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is widely used in oligonucleotide bioanalysis due to its high sensitivity, low detection limits, high selectivity, and specificity. Methods using LC/MS/MS enable the rapid and accurate detection and quantification of low-concentration target compounds in complex matrices without the need for special reagents and probes.[3]

To address the challenges in sample preparation, Agilent developed an automated SPE workstation based on the Bravo liquid handling platform, combined with the next-generation triple quadrupole LC/MS/MS system, the 6495D triple quadrupole LC/MS system, providing a complete solution from sample preparation to data analysis. The 6495D triple quadrupole LC/MS system excels in high sensitivity quantification and low detection limits from complex matrices, while the automated SPE workstation significantly reduces manual intervention time and improves operational consistency and reliability. This complete automated SPE bioanalysis workflow significantly enhances the efficiency and reliability of quantitative oligonucleotide bioanalysis.

### 6495D triple quadrupole LC/MS system

The 6495D triple quadrupole LC/MS system is equipped with 4th generation iFunnel technology. This technology enables high sensitivity data while maintaining system robustness and the durability to handle the most challenging analytes in heavy matrix.

### Automated SPE workstation

Based on traditional manual SPE sample preparation processes, Agilent developed an automated SPE workstation that automates the following steps: blank/internal standard addition, lysis sample buffer addition, SPE conditioning, equilibration, sample loading, two-stage washing, elution, and reconstitution. This process reduces manual intervention time by over 80%, significantly improving work efficiency. Real-time display and recording of the experimental process enhance data accuracy and traceability, while fully automated operation improves the reliability and reproducibility of experimental results.

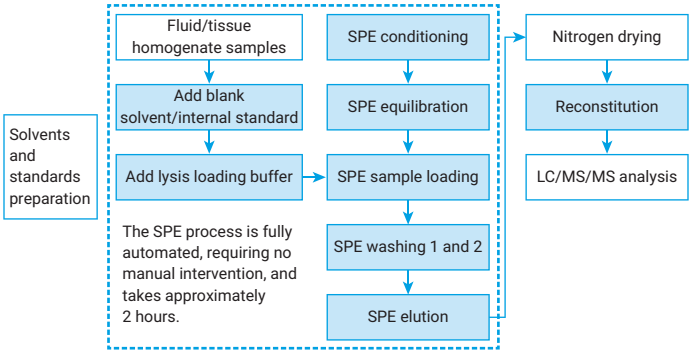


**Figure 2.** Workflow of oligonucleotide bioanalysis based on SPE sample preparation. The parts within the dashed box are fully automated by the SPE workstation, requiring no manual intervention.

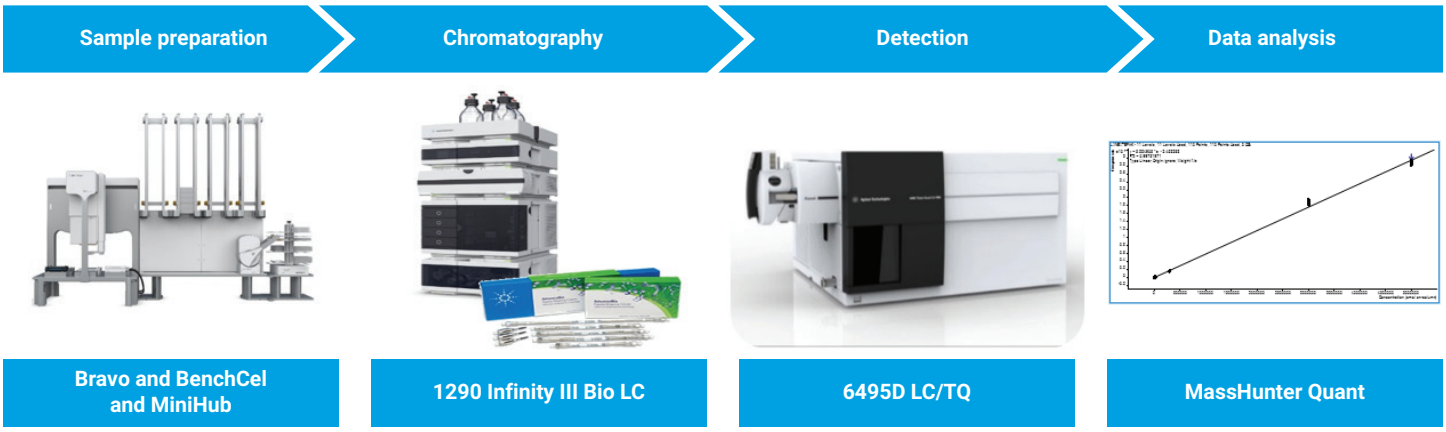


**Figure 1.** Integrated workflow of the Agilent automated SPE bioanalysis.

## Experimental

### Instruments

- Agilent 1290 Infinity III Bio LC
- Agilent 6495D triple quadrupole LC/MS system
- Agilent Automated SPE workstation, including Bravo, BenchCel and MiniHub

### Sample preparation

#### Experimental plates

Oligonucleotide drugs often encounter issues with nonspecific binding. To mitigate this, it is recommended to use low-DNA-binding materials for sample plates, collection plates, and EP tubes. In this application example, we used the Clarity OTX, 25 mg/well, 96-well SPE plate.

#### Solvent preparation

After method optimization, the solvents used in the SPE experiment were determined as shown in Table 1.

Before running the sample preparation procedure, the software prompted pipetting sufficient volumes of solvents and they were placed in the corresponding positions of the automated SPE workstation.

#### Sample preparation

Mipomersen calibration standards and quality control (QC) sample working solutions were prepared using 1% BSA and 0.1% Triton X100 aqueous solution, with concentrations of 0.04 to 20 µg/mL and 0.12 to 16 µg/mL, respectively. The calibration standards and QC samples were diluted with the previously described working solution into blank plasma containing 0.1% Triton X100 at a dilution factor of 20. The calibration standard concentration range was 2 to 1,000 ng/mL, with 8 concentration levels and 2 parallel samples per level. The QC sample concentration range was 6 to 800 ng/mL, with 4 concentration levels and 6 parallel samples per level.

Then we pipetted 100 µL of calibration standards, QC samples, and blank samples into a 96-well plate and placed it in the corresponding position of the automated SPE workstation.

After preparing the solvents, sample plate, and other plates, we ran the Auto SPE protocol to fully automate the following steps: blank solvent and internal standard addition, loading buffer addition, SPE conditioning, equilibration, sample loading, two-stage washing, and elution. Subsequently, we performed nitrogen drying at 40 °C, reconstituted the materials, and proceeded with LC/TQ analysis.

### LC/TQ analysis

#### Optimization of liquid chromatography conditions

Triethylamine (TEA) and diisopropylethylamine (DIPEA) are commonly used ion-pairing reagents that could effectively form ion pairs with oligonucleotides, improving separation efficiency and detection sensitivity. Hexafluoroisopropanol (HFIP) is used as an acid modifier and is typically combined with TEA or DIPEA to further enhance the ion-pairing effect.[3] Through optimization and screening, we selected TEA and HFIP as mobile phase additives.

Additionally, we optimized the concentrations of TEA and HFIP. At initial concentrations of 0.2% TEA and 1% HFIP, adequate peak shapes and responses were obtained. However, after multiple injections of matrix samples, significant peak tailing and carryover issues were observed. To address these issues, we increased the concentrations to 0.4% TEA and 2% HFIP. Although the response decreased, this successfully resolved the peak tailing and carryover issues, meeting the requirements for sample analysis.

Furthermore, we added a step to rinse the chromatography column three times after the analyte elution to further reduce carryover.

**Table 1.** Reagents used in SPE sample preparation.

| Solvent | Composition | Solvent | Composition |
|---|---|---|---|
| Internal Standard | 500 ng/mL mipomersen-3 in 1% BSA 0.1% Triton X100 aqueous solution | Wash buffer 1 | 50 mM $NH_4OAc$ (pH 5.5) aqueous solution |
| Blank Solvent | 1% BSA 0.1% Triton X100 aqueous solution | Wash buffer 2 | 50 mM $NH_4OAc$ (pH 5.5) in 50% ACN aqueous solution |
| Loading Buffer | Clarity OTX lysis-loading buffer | Elution Buffer | 100 mM $NH_4HCO_3$ in $H_2O$ (pH 9.5) / ACN / THF (5:4:1 v/v/v) with 1 mM TCEP |
| Conditioning Solvent | Methanol | Reconstitution solution | 1 mM TCEP 20% Methanol aqueous solution |
| Equilibration Buffer | 50 mM $NH_4OAc$ (pH 5.5) aqueous solution | | |

## LC/TQ parameters

The optimized LC/TQ method parameters were shown in Table 2.

**Table 2.** LC/TQ method parameters for mipomersen.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ion Source Parameters | AJS ESI | | | | | | |
| | Drying Gas Temperature and Flow | 170 °C, 12.0 L/min | | | | | |
| | Nebulizer Gas | 15.0 psi | | | | | |
| | Sheath Gas Temperature and Flow | 370 °C, 12.0 L/min | | | | | |
| | Capillary Voltage (V) | 4,000 | | | | | |
| | Nozzle Voltage (V) | 2,000 | | | | | |
| Acquisition Parameters | **Compound Name** | **Precursor *m/z*** | **Product *m/z*** | **Dwell (ms)** | **CE (V)** | **iFunnel** | **Polarity** |
| | IS | 662.5 | 94.9 | 40 | 32 | Large molecule | Negative |
| | IS | 745.4 | 94.9 | 40 | 47 | Large molecule | Negative |
| | mipomersen | 796.4 | 94.9 | 200 | 57 | Large molecule | Negative |
| | mipomersen | 896.2 | 94.9 | 200 | 31 | Large molecule | Negative |
| | mipomersen | 1,024.3 | 94.9 | 200 | 33 | Large molecule | Negative |

| Parameter | Value |
|---|---|
| Column | Agilent AdvanceBio Oligonucleotide column, 2.1 × 50 mm, 2.7 µm (part number 659750-702) |
| Injection Volume | 10 µL |
| Column Temperature | 50 °C |
| Flow Rate | 0.3 mL/min |
| Gradient | MP A 0.4% TEA, 2% HFIP in $H_2O$<br>MP B 0.4% TEA, 2% HFIP in MeOH<br>Time (min) %A %B<br>0 90 10<br>5 60 40<br>6 60 40<br>6.1 90 10<br>8 10 90<br>8.1 90 10<br>10 10 90<br>10.1 90 10<br>12 10 90<br>12.1 90 10 |
| Stop Time | 15 min |
| Wash Solvent | 50% Methanol/Water |

## Results and discussion

Plasma samples containing mipomersen were processed using the automated SPE workstation and analyzed using the 6495D triple quadrupole LC/MS system.

The results were as follows:

- **Dynamic linear range:** 2 to 1,000 ng/mL, $R^2$ = 0.999.
- **Reproducibility of QC samples:** 4 concentration levels, 6 parallel samples per level, good reproducibility with most RSD less than 5%, and low QC points less than 10%.
- **Accuracy of QC samples:** High accuracy of back-calculated concentrations, all within 85 to 115%.

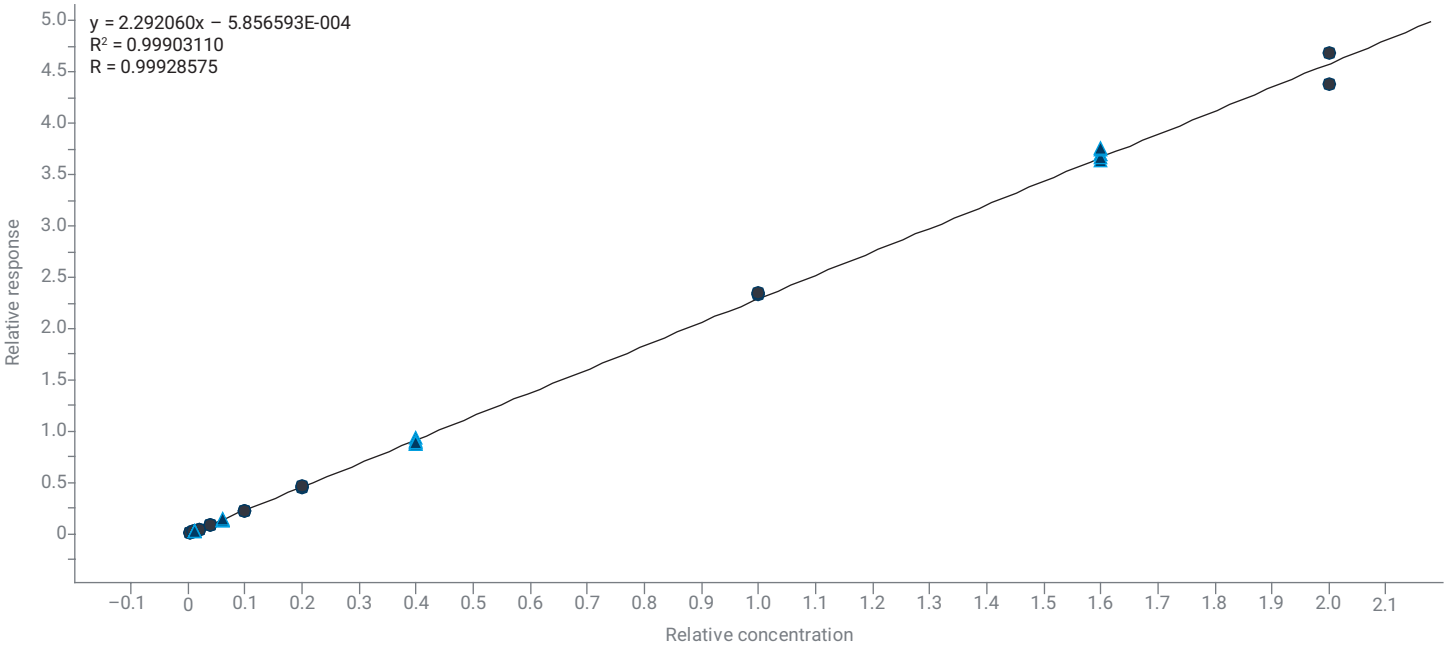


**Figure 3.** Calibration curve of mipomersen in pig plasma, dynamic linear range 2 to 1,000 ng/mL.

**Table 3.** Results of QC samples of mipomersen in pig plasma processed by automated SPE workstation.

| QC Sample | Exp. Conc. (ng/mL) | Cal. Conc. (ng/mL) | Accuracy (%) | %RSD |
|---|---|---|---|---|
| LQC-1 | 6 | 5.13 | 85.52 | 9.43 |
| LQC-2 | | 6.49 | 108.24 | |
| LQC-3 | | 6.75 | 112.50 | |
| LQC-4 | | 6.23 | 103.81 | |
| LQC-5 | | 6.14 | 102.28 | |
| LQC-6 | | 6.64 | 110.67 | |
| GMQC-1 | 30 | 30.79 | 102.64 | 3.25 |
| GMQC-2 | | 29.84 | 99.46 | |
| GMQC-3 | | 30.58 | 101.93 | |
| GMQC-4 | | 28.23 | 94.09 | |
| GMQC-5 | | 28.98 | 96.61 | |
| GMQC-6 | | 29.78 | 99.26 | |

| QC Sample | Exp. Conc. (ng/mL) | Cal. Conc. (ng/mL) | Accuracy (%) | %RSD |
|---|---|---|---|---|
| MQC-1 | 200 | 199.16 | 99.58 | 2.42 |
| MQC-2 | | 192.68 | 96.34 | |
| MQC-3 | | 203.92 | 101.96 | |
| MQC-4 | | 194.51 | 97.25 | |
| MQC-5 | | 191.14 | 95.57 | |
| MQC-6 | | 194.34 | 97.17 | |
| HQC-1 | 800 | 821.06 | 102.63 | 1.26 |
| HQC-2 | | 807.25 | 100.91 | |
| HQC-3 | | 816.19 | 102.02 | |
| HQC-4 | | 801.19 | 100.15 | |
| HQC-5 | | 807.50 | 100.94 | |
| HQC-6 | | 792.78 | 99.10 | |

We evaluated the inter-batch reproducibility of the automated SPE workstation through a three-day repeatability experiment, with QC samples at four concentration levels and six replicates per concentration level.

The experimental results were as follows:

- The RSD for QC samples at four concentration levels was less than 10% each day.
- Over three days, more than 98% of the 72 QC samples across four concentration levels had accuracies between 80 and 120%.
- The back-calculated accuracy of the LLOQ at 2 ng/mL remained stable between 80 and 120%.

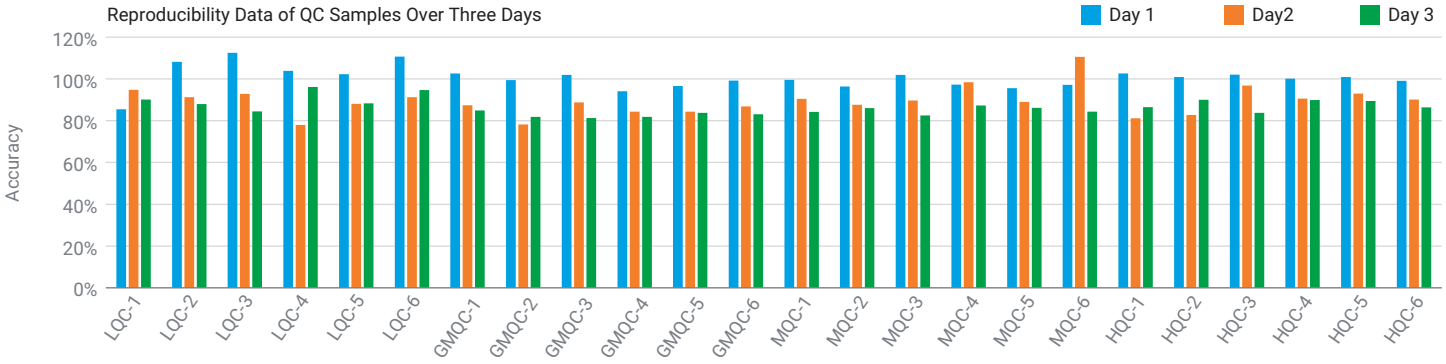


**Figure 4.** Accuracy results of QC samples in a three-day repeatability experiment.

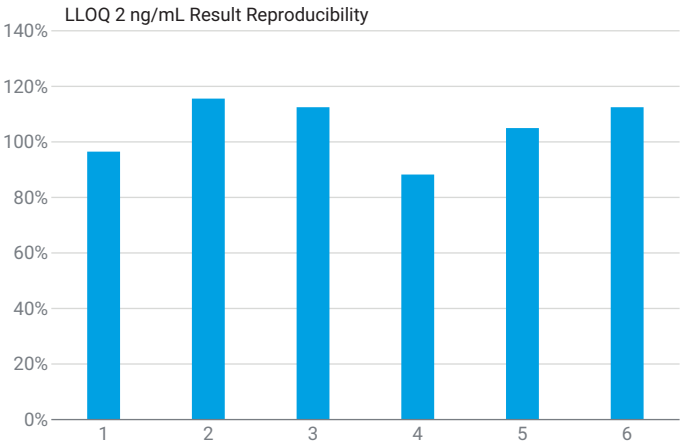


**Figure 5.** Reproducibility of LLOQ results in a three-day repeatability experiment.

**Table 4.** Reproducibility results of QC samples in three-day repeatability experiment.

| QC Level | Conc. (ng/mL) | %RSD | | |
|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 |
| LQC | 6 | 9.43 | 6.12 | 4.44 |
| GMQC | 30 | 3.25 | 4.02 | 1.51 |
| MQC | 200 | 2.42 | 8.54 | 1.84 |
| HQC | 800 | 1.26 | 6.19 | 2.59 |

## Conclusion

This study verified the use of the automated Agilent SPE workstation combined with the Agilent 6495D triple quadrupole LC/MS system for oligonucleotide bioanalysis studies through the quantitative analysis of mipomersen in pig plasma. The experimental results showed that the system exhibited excellent linearity ($R^2$=0.999) within the dynamic range of 2 to 1,000 ng/mL. The reproducibility and accuracy of the QC samples were within acceptable ranges, with most RSD values below 5%, QC low points below 10%, and back-calculated concentration accuracy between 85 and 115%. Additionally, in the three-day repeatability experiment, the inter-batch reproducibility of the QC samples was good, with RSD values all below 10%. The back-calculated accuracies for QC samples at high, medium, and low concentration levels, as well as the LLOQ (2 ng/mL), remained stable between 80 and 120%.

By reducing manual intervention and improving operational consistency, the automated SPE workstation significantly enhances the efficiency and reliability of sample preparation. Combined with the high sensitivity and low detection limits of the 6495D triple quadrupole LC/MS system, this solution achieves efficient and accurate detection of low-concentration oligonucleotides in complex matrices. Overall, this automated solution has broad application prospects in high-throughput oligonucleotide bioanalysis and meets the high requirements for quantitative analysis in drug development.

## References


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