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Nota de aplicación

An Automated, Standardized, Kit-Based Sample Preparation Workflow for Bioanalytical Quantification of Therapeutic Oligonucleotides

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Abstract

Oligonucleotide Therapeutics (ONTs) are a key focus area for many drug developers today given their powerful ability to address disease biology at the level of gene transcription and translation, and for their high target specificity and low toxicity. As the pipeline for this therapeutic class of drugs continues to expand, so does the need for sensitive, accurate, and robust bioanalytical assays to support this drug discovery and development pipeline. LC-MS detection and quantification is a widely accepted technology for bioanalytical studies, for the many benefits it affords (*i.e.*, broad drug applicability, sensitivity, selectivity, and broad linear dynamic range). However, achieving reproducible performance with LC-MS based bioanalytical assays can be challenging. In general, the greatest source of variability for these assays arises from the sample preparation needed to extract the drug and its metabolites from biofluids, and this is especially true for oligonucleotide extractions. Liquid-Liquid Extraction (LLE) and Solid Phase Extraction (SPE) are the two most widely used techniques for the extraction of ONTs from biofluids for LC-MS based quantification. LLE is a low throughput, difficult to automate technique which skilled and experienced scientists to develop, optimize, and implement these methods within a lab or across an organization. SPE is a more automation friendly, higher throughput assay, but may require

systematic optimization of every step to achieve desired recovery, reproducibility, and sensitivity. To this end, a simple, broadly applicable sample preparation workflow for ONTs that reduces the need for method development and brings greater consistency and reproducibility to LC-MS bioanalytical results is therefore highly desired. The OligoWorks™ SPE Microplate Kit (OligoWorks Kit) from Waters has been designed with this in mind. It utilizes standardized, detergent free reagents, and a robust optimized protocol that works across a diverse range of ONTs with little to no method development needed. The automation friendly reagents and SPE devices provided in each kit make it easy to automate the sample preparation procedure on an automated liquid handler, like the Andrew+™ Pipetting Robot, which can further enhance analytical performance and productivity and reduce human error/variability.

This work uses the OligoWorks Kit components and standard protocol (Figure 1) automated on the Andrew+ Pipetting Robot to successfully extract a diverse range of ONTs from plasma and achieve accurate, robust, and reproducible bioanalytical performance, with little to no need for method development.

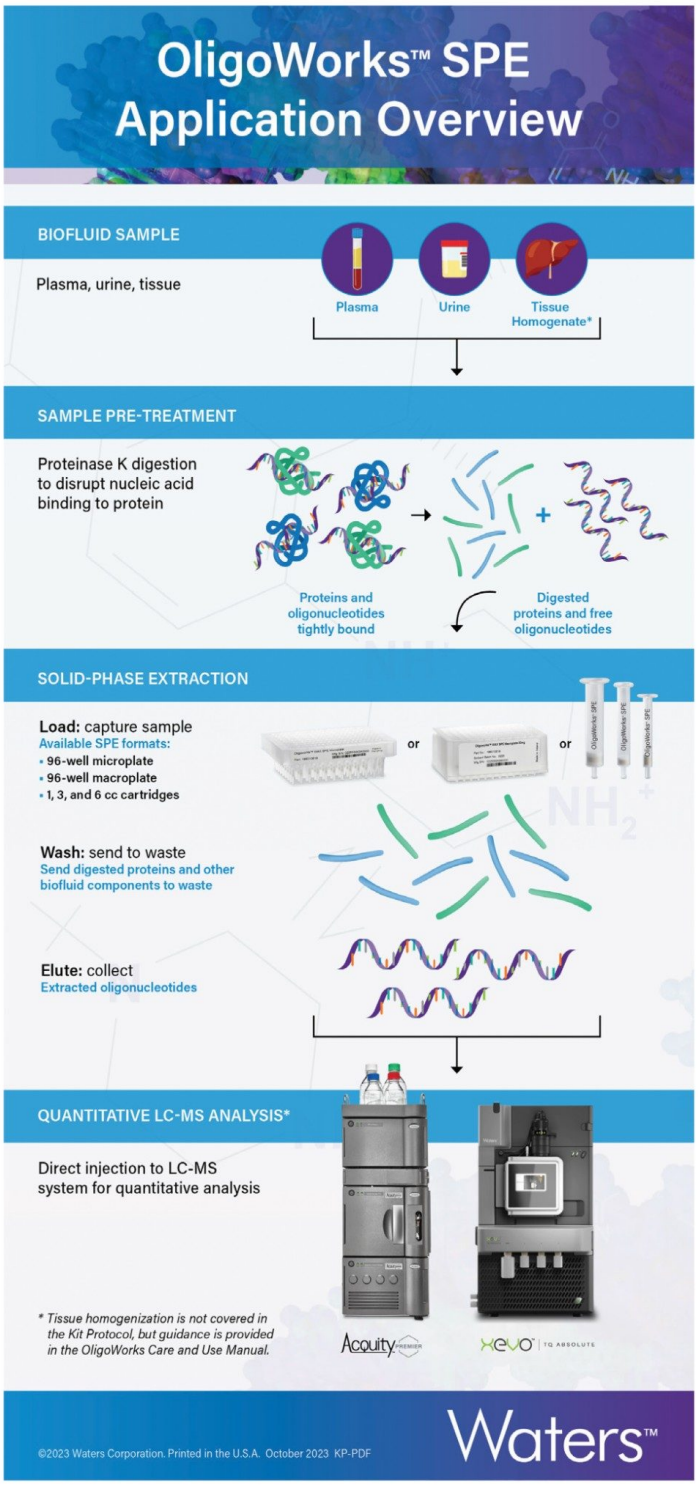

Figure 1. Graphical illustration of oligonucleotide bioanalytical quantification sample

preparation, extraction and LC-MS workflow.

Benefits

- A standardized, detergent free, kit-based solution for the extraction and LC-MS quantification of therapeutic oligonucleotides from biomatrices that requires little to no method development
- Achieve excellent recoveries (>80%) with low %CV (<15%) across a diverse range of ONTs
- Automation friendly workflow as demonstrated with the Andrew+ Pipetting Robot, with Click & Execute OneLab™ Software Library Methods that make implementation easy and improve assay performance
- Accurate, sensitive, and reproducible quantification across a diverse set of therapeutic oligonucleotides from extracted plasma samples

Introduction

The Solution

OligoWorks Kits are simple, standardized, flexible, and automation friendly sample preparation kits designed to enable accurate and robust LC-MS based bioanalytical quantitation across a diversity of oligonucleotides. The kits use an effective enzyme-based digestion sample pretreatment step with RapiZyme™ Proteinase K Digestion module to effectively disrupt oligonucleotide-biomatrix protein binding followed by selective purification using the OligoWorks SPE device, which contains a mixed-mode anion exchange SPE sorbent, designed, and QC verified for oligonucleotide performance. Each kit contains pre-measured, lot traceable, detergent free reagents, and a universal protocol to streamline the oligonucleotide sample preparation workflow and facilitate implementation by users at all experience levels.

The goals of this work were to demonstrate efficient extraction and accurate quantification of oligonucleotides from plasma using the OligoWorks Microplate Kit, automated on the Andrew+ Pipetting Robot. For this evaluation, gene-expression modulator 91 (GEM91), a 25-mer phosphorothioated antisense oligonucleotide (MWT 7771), GEM 132, a 20-mer phosphorothioated antisense oligonucleotide with 2′ methoxy caps (MWT 6600), a N-Acetylalactoseamine (GalNAc) conjugated siRNA (MWT 8590), and a 20-mer single-stranded DNA (ssDNA)

oligonucleotide (MWT 6122) were used.

Experimental

LC-MS Chromatographic Separation and Experimental Conditions

LC system:	ACQUITY™ Premier UPLC System with FTN
Column:	ACQUITY Premier Oligonucleotide C_{18} Column, 130 Å, 1.7 µm, 2.1 x 50 mm, 1/pk (p/n: 186009484)
Column temperature (°C):	55 °C
Sample temperature (°C):	10 °C
Mobile phase A:	1% HFIP (Hexafluoro-2-propanol) 0.1% DIPEA (N, N-Diisopropylethylamine) in H_2O
Mobile phase B:	0.75% HFIP (Hexafluoro-2-propanol), 0.0375% DIPEA (N, N-Diisopropylethylamine, 65% ACN 35% H_2O
Purge solvent:	25:25:25:25 Methanol:Acetonitrile:Isopropanol:Water
Injection volume (µL):	10 µL

LC Gradient table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.600	95	5	6
3.25	0.600	77	23	6
3.75	0.600	10	90	6
4.10	0.600	10	90	6
4.25	0.600	95	5	6

MS System Conditions

MS system:	Xevo™ TQ Absolute MS
Ionisation mode:	ESI Negative
Acquisition mode:	MRM
Capillary voltage (kV):	3
Desolvation temperature (°C):	600
Desolvation gas flow (L/Hr):	1000
Cone gas flow (L/Hr):	150
Collision gas flow (L/Hr):	0.2
Nebulizer (Bar):	7

MRM Transitions

MRM transitions				
Oligonucleotide	**Precursor (*m/z*)**	**Product (*m/z*)**	**Cone voltage (V)**	**Collison energy (eV)**
GEM91	646.6	95.0	40	30
GEM132	824.5	94.9	40	40
GalNAc	714.6	227.4	40	20
ss DNA (20-mer)	764.3	125.1	40	30

Data Management

Instrument control software:	MassLynx™ (v4.2)
Quantification software:	TargetLynx™ (v4.2)
Automation software:	OneLab (1.19.2)

Chemicals, reagents, materials and solvents

GEM91 and GEM132 were sourced from Avecia Nitto Denko (MA, USA), GalNAc conjugated siRNA was kindly donated by Alnylam Pharmaceuticals (Cambridge, MA). The ssDNA 20-mer oligonucleotide was sourced from Waters Corporation (Milford, MA).

MS grade Methanol, water, acetonitrile, isopropanol, Hexafluoro-2-propanol (HFIP), N,N-Diisopropylethylamine (DIPEA) and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO, USA). K_2 EDTA rat plasma was procured from BioIVT (Westbury, NY, USA). DNase/RNase-free distilled water was purchased from ThermoFisher Scientific (p/n: 10977015) and was used for oligonucleotide standard preparation and SPE sample eluate dilution. OligoWorks Kit (p/n: 186010614 <https://prod1-author.waters.com/nextgen/global/shop/application-kits/186010614-oligoworks-spe-microplate-kit.html>) was procured from Waters Corporation (Milford, MA, USA).

OligoWorks Kit Wash Reagent Preparation

OligoWorks Kit SPE Wash 1: 50 mM Ammonium Acetate buffer, pH 5.5 was prepared by weighing out 3.84 g ammonium acetate and bringing to 1 Liter volume and adjusting pH to 5.5.

OligoWorks Kit SPE Wash 2: 30% Methanol/70% Water solution was prepared by adding 300 mL of methanol to 700 mLs of water.

Stock solutions, Calibration Curve, and QC Sample Preparation

GEM91, GEM132, GalNAc conjugated siRNA, and ssDNA were reconstituted in RNase/DNase-free distilled Water to provide a 1 mg/mL stock solution using Eppendorf DNA LoBind™ Tubes (p/n: 022431021 and 022431005). A combined working stock solution for all four oligonucleotides at 10 µg/mL each was created by adding 10 µL of each of the 1 mg/mL stock solution to 960 µL of water in DNA LoBind tubes. Calibration curve (0.25–1000 ng/mL) and quality control (QC) samples (LQC-0.75 ng/mL, MQC-50 ng/mL and HQC-750 ng/mL) in plasma were prepared using the Andrew+ Pipetting Robot.

Sample Pretreatment and SPE Extraction using the OligoWorks Microplate Kit

Prepared calibration curve and QC samples (100 µL) were added to an Eppendorf 1mL deep well plate and digested using the reagents and protocol supplied in the RapiZyme Proteinase K Digestion Module and subsequently extracted using the OligoWorks Kit SPE Microplate and eluent, following the protocol provided in the OligoWorks Kit and OligoWorks care and use manual (720008066 < https://www.waters.com/waters/support.htm?lid=135127508>). This protocol is illustrated in Figure 2. (Note: reagent volume of Proteinase K in the OligoWorks kit is sufficient to automate a full plate of 96 samples with 10% overage. If higher overage is desired, additional RapiZyme Proteinase K Digestion Module (p/n: 186010601 < https://www.waters.com/nextgen/global/shop/standards--reagents/186010601-rapizyme-proteinase-k-digestion-module.html>) can be procured separately.)

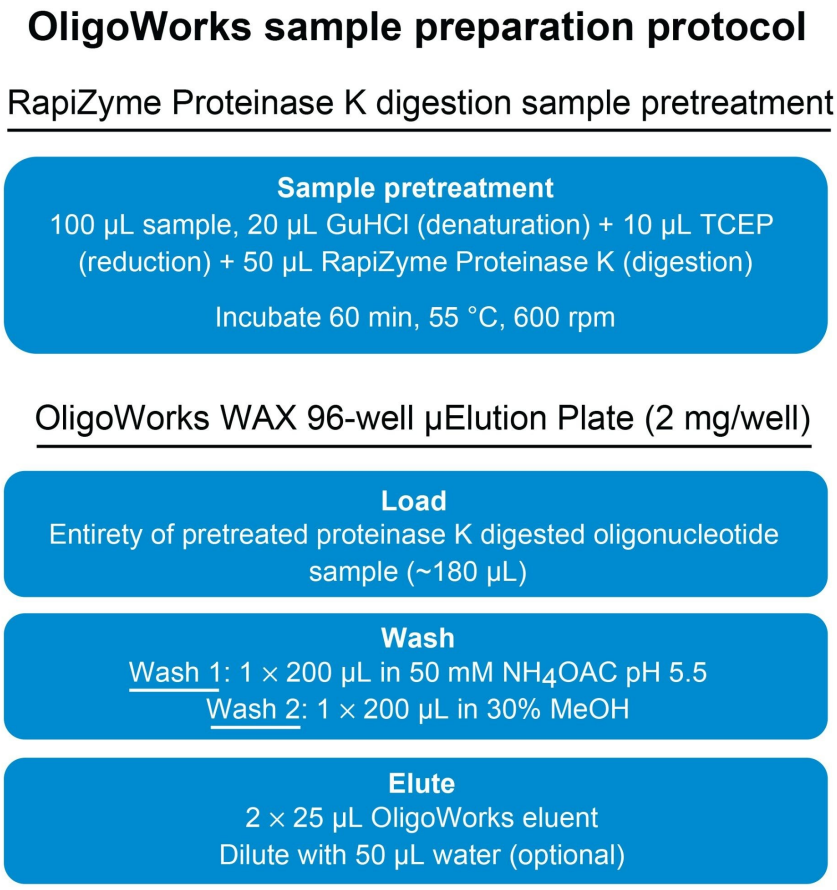

Figure 2. Graphical representation of the OligoWorks Kit Protocol (p/n: 186010614), optimized for 100 µL starting plasma/sera sample.

Automation platform

Andrew+ Pipetting Robot was used to generate calibration curves and QC's of plasma samples in a Waters QuanRecovery 700 µL plate by downloading and modifying the Simple Serial Dilution Preparation < https://onelab.andrewalliance.com/app/lab/GK6ovDkA/library/simple-serial-dilution-preparation-9jn2GGwa> method from the OneLab Software Library. All calibration curves and QC's were then extracted in triplicate by downloading the Click & Execute OligoWorks RapiZyme Proteinase K Digestion method (Figure 3A) and OligoWorks WAX SPE Microplate method (Figure 3B) from the OneLab Software Methods Library. The complete workflow, from creating plasma calibration curves and QC samples to digesting and extracting the oligonucleotides with the OligoWorks Microplate Kit was fully automated on Andrew+ Pipetting Robot

configured with the Heater-Shaker+ and Extraction+ Connected Devices.

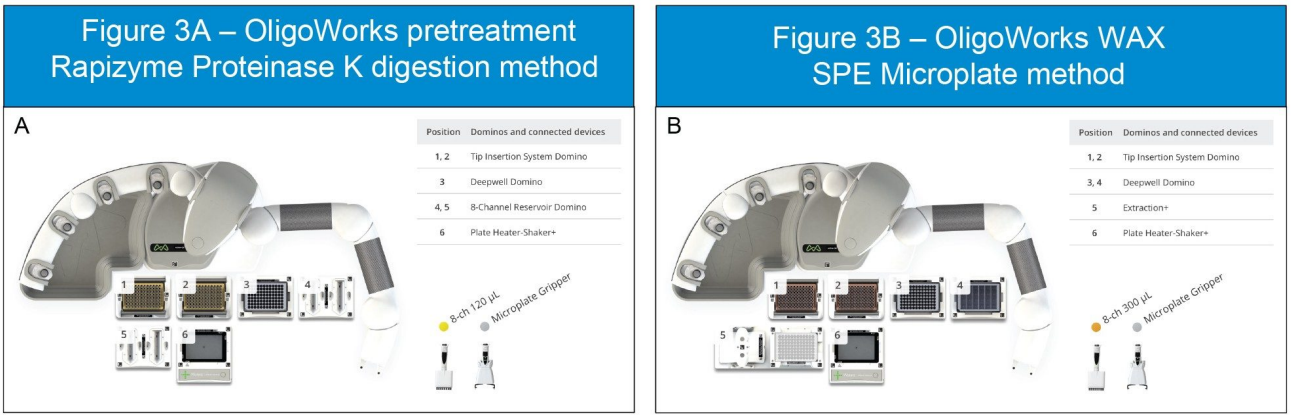

Figure 3. Representative Andrew+ Pipetting Robot Deck Layouts for oligonucleotide sample pretreatment using a Proteinase K Digestion (A) and OligoWorks WAX SPE 96-well Microplate (B). Both layouts illustrate the placement of all dominos, connected devices, and appropriate pipettes required for execution of these methods.

Results and Discussion

Therapeutic Oligonucleotides have proven to be very effective therapies for certain types of genetic or translational dysregulation diseases. With the increase in interest in exploring this therapeutic class for a variety of clinical conditions comes the need for simple, accurate, and robust analytical techniques to analyze and quantify these molecules. Achieving efficient and reproducible extraction of these analytes from complex biological matrices with high recoveries, using relatively simple sample preparation protocols is critically important for LC-MS quantification. Many ADME/DMPK workflows are highly automated for increased efficiency and reproducibility. Liquid-liquid extraction (LLE)-SPE is commonly used for extraction of oligonucleotides from biological matrices. Although effective, LLE is a slow, low-throughput manual process that is not easily automatable or scalable. Other commercially available SPE kits for this workflow use detergent-based reagents, which require extensive washing during SPE to remove these detergents and often require evaporation and reconstitution before injection into LC-MS systems to ensure SPE eluent compatibility. These steps add time, and often increase assay variability with the potential of oligonucleotide loss due to adsorption, solubility, and

potential degradation.

In contrast the OligoWorks Kit-based solution utilizes a simple, detergent-free workflow for LC-MS quantification of ONTs from biofluids that works well across a diverse range of ONTs with little to no method development, and as demonstrated in this study, is easily automated. Sample pretreatment with the RapiZyme Proteinase K Digestion Module effectively disrupts the strong oligonucleotide protein binding that occurs in biological fluids without the use of detergents, thereby removing the need for extensive washing and dry down steps prior to LC-MS analysis. The OligoWorks WAX SPE sorbent is designed to selectively bind oligonucleotides and wash away unwanted matrix components contained within the sample, resulting in a clean SPE eluate that can be directly injected onto an LC-MS system. As described in application note 720008086, the OligoWorks solution demonstrates excellent performance, with high recovery and repeatability across a diverse range of oligonucleotides and with various starting biological sample volumes. The Click and Execute OneLab Software Library Methods for OligoWorks sample pretreatment & SPE enable rapid method deployment, execution, and scalability while lowering risk of human error thus enhancing reproducibility and enabling robust analytical performance. Use of the OligoWorks Kit starting protocol, fully automated on the Andrew+ resulted in excellent oligonucleotide recovery from plasma (>96%) with less than 5% difference seen between manual and automated sample processing as illustrated in Figure 4.

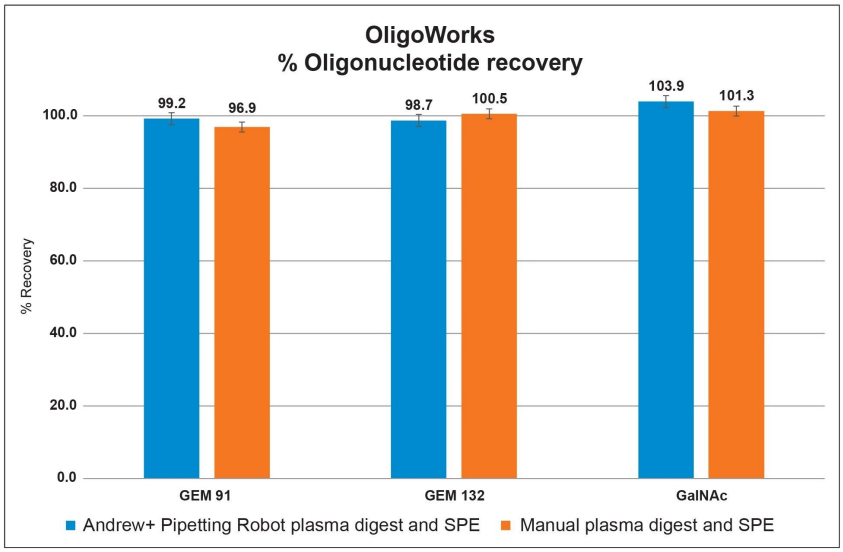

Figure 4. Comparable automated (Andrew+ Pipetting Robot) vs manual sample preparation and extraction performance using the OligoWorks Kits for GEM91, GEM132, and GalNAc oligonucleotides with >96% recovery and <5% difference from manual to automated sample processing ensuring a fit-for-purpose automated OligoWorks sample preparation and extraction solution.

The OligoWorks kit utilized in this study required no method development and facilitated the accurate quantification of four oligonucleotide therapies yielding excellent quantitative performance (no internal standard correction) from 100 µL plasma samples. Automating the workflow on the Andrew+ Pipetting Robot, lower limits of quantification (LLOQ's) of 250 pg/mL for GEM91, GEM132, and GalNAc conjugated siRNA, and 0.50 ng/mL for ssDNA 20-mer oligonucleotides were observed. Calibration curves were linear (r^2>0.99) from 0.25–1000 ng/mL (GEM91, GEM132, and GalNAc-siRNA) and 0.5–1000 ng/mL (ss DNA), with % bias and coefficient of variations (CV's) <15% for all triplicate points at each level achieving the recommended small molecule bioanalytical method validation criteria (as shown in Table 1). Specifically, accuracies and CVs across the calibration curves for GEM91, GEM 132, GalNAc, and ss DNA ranged from 85.2–119.2% and 1.97–13.87%, respectively.

Accuracy and precision for all QC levels across triplicate extractions was also within the bioanalytical method validation guidelines of ±15%. Mean accuracies for QC points for GEM91, GEM132, GalNAc conjugated siRNA, and ssDNA 20-mer oligonucleotides were between 92.30–104.07% with mean CVs between 2.82–6.77%,

respectively (as shown in Table 1). Area response for QC points increased linearly across the concentration range, as illustrated in Figure 5.

Sensitive, linear, accurate and precise

Calibration curve statistics

Analyte	Range	Weighting	Linear regression	% Accuracy range	% CV range
GEM91	0.25–1000 ng/mL	1/x	>0.99	85.4–114.7	2.01–11.43
GalNAc				85.2–114.4	2.01–13.44
GEM132				85.9–119.2*	1.97–9.67
ss DNA (20-mer)	0.50–1000 ng/mL			85.7–112.4	0.99–13.87

*% Accuracy of 119.2 for LLOQ - Acceptable per Bioanalytical method validation guidelines

QC statistics

Analyte	QC level	Expected concentration (ng/mL)	Mean observed concentration (ng/mL) (N=3)	Mean % accuracy (N=3)	Mean % CV (N=3)
GEM91	LQC	0.75	0.74	98.17	6.42
GalNAc			0.69	92.30	2.90
GEM132			0.78	104.07	5.13
ss DNA (20-mer)			0.69	92.63	8.69
GEM91	MQC	50	52.97	105.95	2.82
GalNAc			49.79	99.56	6.42
GEM132			51.72	103.43	7.41
ss DNA (20-mer)			55.15	110.29	0.99
GEM91	HQC	750	756.55	100.87	4.61
GalNAc			733.97	97.87	13.44
GEM132			748.28	99.82	6.77
ss DNA (20-mer)			763.63	101.84	4.66

Table 1. Linear accurate and precise quantitation calibration curve sample (A) and QC sample (B) performance statistics for GEM91, GEM132, GalNAc, and ss-DNA 20-mer oligonucleotides from plasma, using the OligoWorks kit, automated on the Andrew+ Pipetting Robot followed by and subsequent LC-MS/MS analysis.

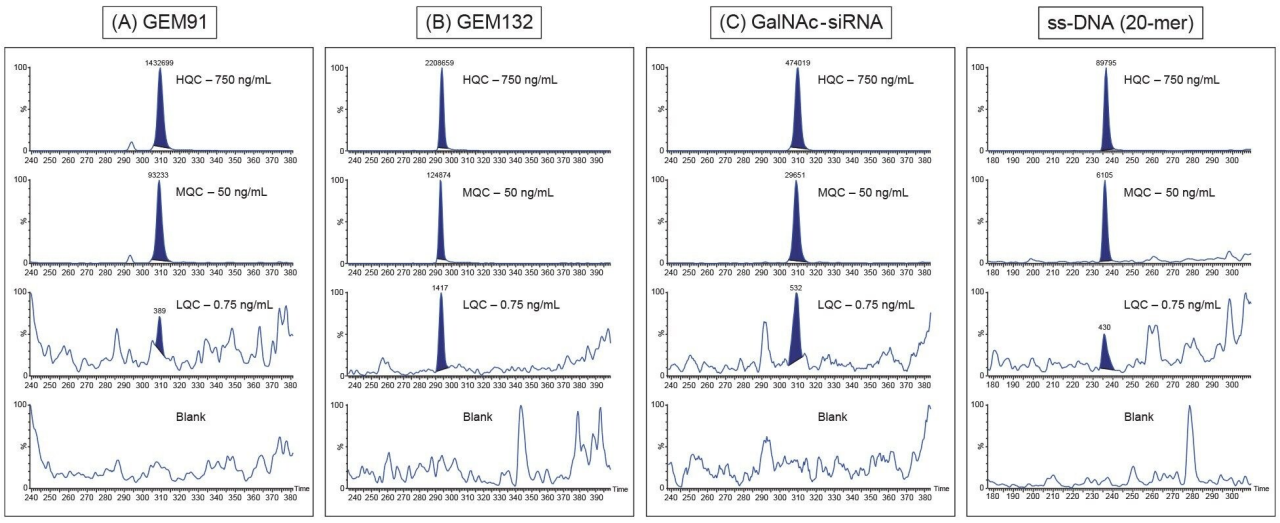

Figure 5. Representative QC chromatograms for GEM91 (A), GEM132(B), GalNAc (C), and ss DNA (D).

Conclusion

Accurate and robust oligonucleotide quantification from plasma was achieved using the OligoWorks SPE Microplate kit (with simple stepwise protocols and standardized, pre-measured, detergent free reagents). This workflow was fully automated on the Andrew+ Pipetting Robot with downloadable OneLab Click & Execute Library Methods to facilitate easy and reproducible execution of the OligoWorks Kit sample preparation and extraction workflow from day-to-day, user-to-user, and lab-to-lab. This fully automated and standardized approach (achieving high oligonucleotide recovery) greatly simplified and streamlined sample extraction, maximized lab productivity, reduced errors, and ensured overall analytical method performance.

References

1. Margot Lee, Nikunj Tanna, Mary Trudeau. Development of a Standardized, Kit-Based Approach for Selective and Reproducible Sample Preparation and Extraction for Therapeutic Oligonucleotides from Biological

Matrices, Waters Application Note 720008086, September 2023.

2. OligoWorks SPE Kits and Components, Waters User Manual, 720008066 <https://www.waters.com/waters/support.htm?lid=135127508> .

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