

Development of a Standardized, Kit-Based Approach for Selective and Reproducible Sample Preparation and Extraction for Therapeutic Oligonucleotides from Biological Matrices

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Apenas para utilização em pesquisas. Não se destina à utilização em procedimentos de diagnóstico.

Abstract

Oligonucleotide bioanalytical sample preparation workflows are often time consuming, complex, and require extensive method development to achieve adequate extraction efficiency and reproducibility. The margin for error is low and potential variability from day-to-day, user-to-user, and across labs can be very high given the biological nature of each sample and the diversity of oligonucleotide therapeutics now in development. The poor extraction efficiency and reproducibility in oligonucleotide quantification analytical data and general lack of expertise strongly support the need for a standardized, kit-based approach for oligonucleotide bioanalytical sample preparation and extraction.

Benefits

- A standardized, streamlined approach to oligonucleotide bioanalytical sample preparation using the OligoWorks WAX SPE Microplate Kit Solution
 - Simplified sample preparation with pre-measured, QC-verified, lot-traceable reagents
 - Optimized protocol that minimizes sample preparation time and method development
 - A flexible and scalable kit-based approach that achieves consistently high oligonucleotide recoveries; >80% across a diversity of biomatrices and starting sample volumes (12.5–300 µL)
 - Robust and reproducible performance with RSDs ≤15% for oligonucleotide plasma recoveries
 - Direct LC-MS sample analysis, with injection volumes up to 30 µL, requiring no sample evaporation or reconstitution
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Introduction

Oligonucleotide therapies represent a growing class of therapeutics with tremendous potential given their ability to address disease conditions at the level of gene transcription and translation. As such, the desire and need for their LC-MS bioanalytical quantification in support of their development is increasing. One of the greatest sources of variability in bioanalytical assay arises from the sample preparation. This is especially true for oligonucleotides, where a variety of sample preparation techniques are currently being used, each with multiple protocol steps and varying degrees of oligonucleotide extraction efficiency that increase the margin for error and variability in assay performance. And while assay optimization can bring down this error and enhance reproducibility, the transferability of these methods from user-to-user, or lab-to-lab (*e.g.*, from sponsor-to-CRO) can be quite challenging given the stringent regulatory guidelines and reproducibility requirements needed for valid quantification measurements. All of this underscores the strong need for a simpler, more standardized and streamlined sample preparation workflow that can be readily deployed across labs with varying degrees of scientific expertise, enabling consistently high oligonucleotide recoveries and reduced variability.

In this work we have used the OligoWorks SPE Microplate Kit (p/n: [186010614 < https://www.waters.com/nextgen/global/shop/application-kits/186010614-oligoworks-spe-microplate-kit.html>](https://www.waters.com/nextgen/global/shop/application-kits/186010614-oligoworks-spe-microplate-kit.html)), to simplify and streamline the bioanalytical sample preparation process using the protocol provided with the OligoWorks SPE Kit and supplied reagents for a diversity of therapeutic oligonucleotides in plasma and urine biomatrices. Key performance attributes evaluated include: speed and efficiency of oligonucleotide-protein

binding disruption with Proteinase K digestion sample pretreatment, flexibility and scalability of the standardized protocol, linear MS response across sample volumes from 12.5–300 μL , direct LC-MS assay compatibility of the extracted sample removing the need for sample evaporation and reconstitution, and of course, high extraction efficiency and reproducibility for a diversity of oligonucleotides.

Experimental

For OligoWorks SPE Kit development and evaluation several oligonucleotides were prepared in Sprague Dawley rat plasma K3EDTA or urine purchased from BIIOIVT (Westbury, NY). These oligonucleotides are listed below.

1. MassPREP Oligonucleotide Standard, which contains an oligonucleotide deoxythymidine nucleotide (dT) mix, containing equal amounts of 15 (MWT 4499), 20 (MWT 6020), 25 (MWT 7541), 30 (MWT 9062), and 35 nucleotides (MWT 10584)
2. Gene expression modulator 91 (GEM91), a 25-mer phosphorothioated antisense oligonucleotide (MWT 7771), synthesized by Nitto Denko AVECIA (Milford, MA)
3. GEM 132, a 20-mer phosphorothioated antisense oligonucleotide with 2' methoxy caps (MWT 6600), synthesized by Nitto Denko AVECIA (Milford, MA)
4. An N-Acetylgalactosamine (GalNAc) conjugated -siRNA oligonucleotide (MWT 8590), and a single-stranded DNA (ssDNA), 20-mer oligonucleotide (MWT 6122), generously provided by Alnylam Pharmaceuticals (Cambridge, MA)
5. A lipid-conjugated 16-mer antisense oligonucleotide with 5' palmitate modification, a phosphorothioated backbone and terminal methoxy ethyl modifications (MWT 5726), Biosearch Technologies, Lystrup, Denmark)

For each oligonucleotide evaluated, concentrations in plasma and/or urine were between 0.1–10 $\mu\text{g}/\text{mL}$ and/or 0.01–1 $\text{pmol}/\mu\text{L}$. Starting sample volumes used for OligoWorks SPE Kit development and evaluation were between 12.5–300 μL , with 100 μL being the optimal starting volume when using the OligoWorks SPE Microplate Kit.

Sample Pretreatment and SPE Extraction

Oligonucleotide biological sample aliquots were prepared in quadruplicate and were added to an Eppendorf 1

mL Deepwell plate ([Eppendorf Deepwell Plates - Eppendorf US <https://www.eppendorf.com/us-en/eShop-Products/Laboratory-Consumables/Plates/Eppendorf-Deepwell-Plates-p-PF-55960>](https://www.eppendorf.com/us-en/eShop-Products/Laboratory-Consumables/Plates/Eppendorf-Deepwell-Plates-p-PF-55960)) and digested using the RapiZyme Proteinase K Module contained in the OligoWorks SPE Microplate Kit as referenced in the OligoWorks SPE Kit Care and Use Manual (720008066 [<https://www.waters.com/waters/support.htm?lid=135127508>](https://www.waters.com/waters/support.htm?lid=135127508)). This protocol is illustrated in Figure 1. The reagent volumes used were optimized for 100 μ L of starting biological sample volume (Note: recommended reagent volumes for starting sample volumes from 12.5 to 300 μ L's can be found in the OligoWorks SPE Kit Care & Use Manual). During development of the protocol provided with the OligoWorks SPE Kit it was found that a 1:1 dilution with water of the post-RapiZyme Proteinase K digested sample prior to SPE microplate loading yielded maximum recovery for some of the oligonucleotides (MassPREP OSTs, and GalNac). This 1:1 post-digested sample dilution reduces the total guanidine concentration, which can compete for anion exchange (AX) binding sites and limit oligonucleotide binding capacity, hence the improved recovery of some oligonucleotides when this dilution is implemented.

OligoWorks sample preparation and SPE extraction protocol

RapiZyme proteinase K digestion sample pretreatment

Sample pretreatment

100 μ L biological sample, 20 μ L GuHCl (denaturation) + 10 μ L TCEP (reduction) + 50 μ L RapiZyme proteinase K (digestion)

Incubate 60 min, 55 $^{\circ}$ C, 600 rpm

OligoWorks SPE microplate (2 mg/well)

Load

Entirety of pretreated proteinase K digested oligonucleotide sample (~180 μ L)

Wash

Wash 1: 1 \times 200 μ L in 50 mM NH_4OAC pH 5.5

Wash 2: 1 \times 200 μ L in 30% MeOH

Elute

2 \times 25 μ L OligoWorks eluent
dilute with 50 μ L water (optional)

Figure 1. Graphical representation of the OligoWorks Bioanalytical Sample Preparation Microplate Kit Protocol (p/n: 186010614), optimized for 100 μ L starting plasma/sera sample.

OligoWorks SPE Wash Reagents (Not included in OligoWorks SPE Kits)

OligoWorks SPE Wash Reagent 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 SPE Wash Reagent 2, 30% Methanol/70% water solutions, was prepared by adding 300 mLs of methanol to 700 mLs of water.

LC-MS Analysis

An ion-pairing, reversed-phase ultra-performance liquid chromatographic (UPLC) separation followed by mass spectrometry (MS) detection using multiple reaction monitoring (MRM) on a tandem/triple quadrupole mass spectrometer was employed for oligonucleotide analysis. An ACQUITY Premier BEH C₁₈ 1.7 μm Column (p/n: 186009452 <<https://www.waters.com/nextgen/global/shop/columns/186009452-acquity-premier-beh-c18-column-17--m-21-x-50-mm-1-pk.html>>) was used with a 5-minute analysis time using recommended gradient and MS conditions provided in the OligoWorks SPE Kit Care & Use Manual. MRM MS transitions for each oligonucleotide evaluated are shown in Table 1.

Oligonucleotide	Precursor (m/z)	Fragment (m/z)
OST 15T	642.0	303.0
OST 30T	646.4	303.0
OST 20T	668.1	303.0
OST 25T	684.7	303.0
OST 35T	704.6	303.0
GEM132	732.8	94.9
GEM132	824.5	95.0
GALNAC	779.6	227.2
GEM91	863.1	95.0
Lipid conjugate	1431.8	1536.3

Table 1. MS MRM transitions for each oligonucleotide evaluated for OligoWorks Kit development.

Results and Discussion

Over the past decade, the focus on oligonucleotide therapeutics research and development has greatly increased, putting a great demand for their accurate and reproducible LC-MS quantification from biological matrices in support of their research and development. However, the development of high sensitivity assays

remains challenging with a multitude of sample preparation and extraction choices and options, all varying in their time, complexity and analytical performance. In this application, we have used the OligoWorks SPE Microplate Kit to simplify and streamline the bioanalytical sample preparation for oligonucleotides.

Development and Optimization of the Protocol Provided with the OligoWorks SPE Kit

For the development of the OligoWorks Kit, a broad range of chemically diverse oligonucleotides (listed in the experimental section) were evaluated, with the goal of maximizing oligonucleotide recovery across modalities and biological matrices, resulting in the protocol provided with the OligoWorks SPE Kit. Areas of optimization focused on sample pretreatment and the SPE protocol. A common sample pretreatment for biological matrices is the use of lysis or denaturing buffers. In our hands, it was limited in its extraction efficiency, requiring individual oligonucleotide sample pretreatment optimization. In addition, the downstream SPE extraction of these lysis buffer containing samples required additional large volume washes to effectively remove the buffers and detergents contained in the sample prior to LC-MS analysis. At the same time, use of the lysis buffer restricted sample loading volume to ≤ 25 μL starting plasma sample due to capacity limitations. Essentially, the salts and ionic detergents in this sample compete with the oligonucleotide for anion-exchange retention, resulting in the oligonucleotides loss on loading (break-through) and results in low SPE recovery. For this reason, our approach focused on a proteinase K sample digestion pretreatment strategy, as it provides more uniform disruption of oligonucleotide-protein binding and is detergent-free, eliminating the issues with extensive SPE washing and low (≤ 25 μL) starting sample volumes. Elements of the proteinase K digestion sample pretreatment optimization focus were the 1. specific digestion reagents and their respective concentration, 2. digestion time, and 3. temperature required to achieve maximum digestion efficiency for full oligonucleotide-protein binding disruption (greatest oligonucleotide recovery). Figure 2, panels A-D highlight these evaluations digesting 100 μL of starting plasma sample containing various oligonucleotides followed by LC-MS analysis. Figure 2A demonstrates best oligonucleotide recovery using guanidine as the denaturant and the reductant tris (2-carboxyethyl)phosphine (TCEP), achieving $>87\%$ recovery (1 hour digestion at 55 $^{\circ}\text{C}$) for the GEM91, GEM 132, GalNAc, and lipid conjugated oligonucleotides. While dithiothreitol (DTT) provided similar performance, TCEP was chosen for its liquid and room temperature stability. Figure 2B demonstrates improved oligonucleotide recovery ($\sim 15\%$) for the GEM91, GEM132, and GalNAc oligonucleotides with the addition of 2X the amount of proteinase K enzyme solution (20 mg/mL) using a 15 minute digestion at 65 $^{\circ}\text{C}$. This correlates to a 25 vs 50 μL proteinase K reagent addition, respectively. Digestion temperatures of 45, 50, 55, and 65 $^{\circ}\text{C}$ were chosen for evaluation, as these were commonly reported digestion temperatures for proteinase K. Optimal digestion temperature (best

oligonucleotide recovery) was determined to be 55 °C for the GEM91, GEM132, GalNAc, and lipid conjugated oligonucleotides. This is illustrated in Figure 2C. A digestion temperature of 65 °C was evaluated in attempts to accelerate digestion time (data not shown). While 65 °C provided high oligonucleotide recovery, it resulted in high inter and intra-assay reproducibility. Lastly, a digestion time of one hour provided the best balance of recovery and reproducibility for the diversity of oligonucleotides screened. This performance is highlighted for the GEM91, GEM132, GalNAc, and lipid conjugated oligonucleotides in Figure 2D.

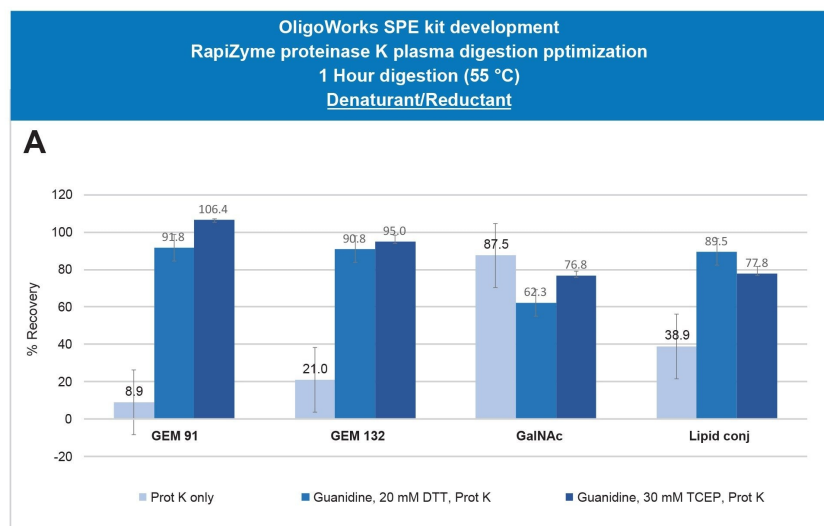


Figure 2. RapiZyme Proteinase K digestion sample pretreatment optimization of the denaturant/reductant reagents (A), Proteinase K enzyme concentration (B), digestion temperature (C), and digestion time (D).

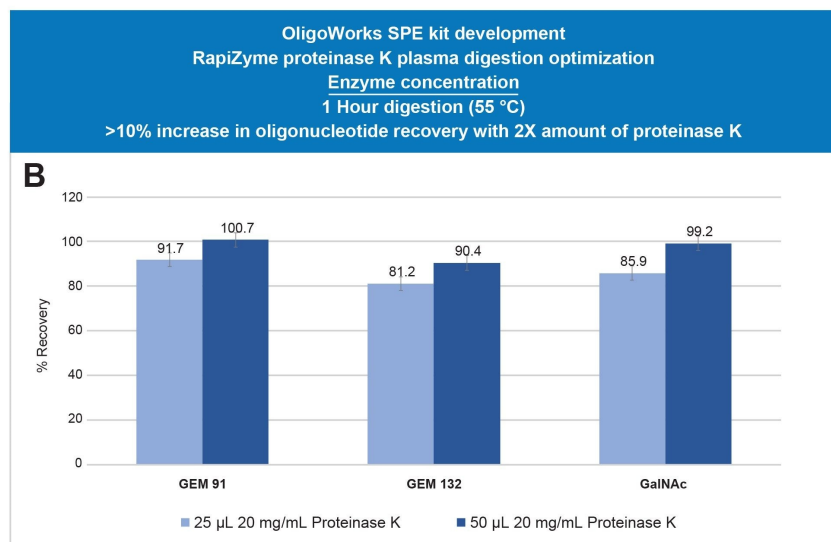


Figure 2. RapiZyme Proteinase K digestion sample pretreatment optimization of the denaturant/reductant reagents (A), Proteinase K enzyme concentration (B), digestion temperature (C), and digestion time (D).

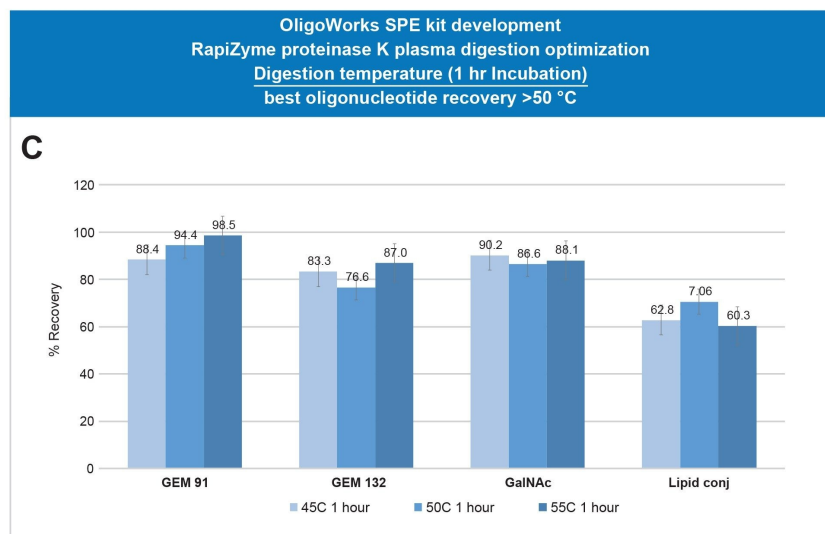


Figure 2. RapiZyme Proteinase K digestion sample pretreatment optimization of the denaturant/reductant reagents (A), Proteinase K enzyme concentration (B), digestion temperature (C), and digestion time (D).

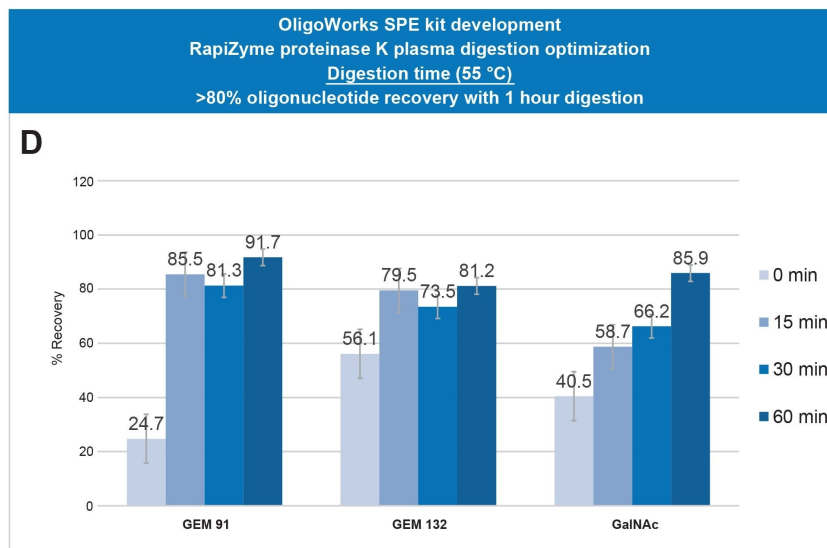


Figure 2. RapiZyme Proteinase K digestion sample pretreatment optimization of the denaturant/reductant reagents (A), Proteinase K enzyme concentration (B), digestion temperature (C), and digestion time (D).

Microplate WAX SPE Protocol Optimization

Following the proteinase K digestion pretreatment optimization, efforts focused on achieving maximum SPE recovery for the diversity of oligonucleotides prepared in various matrices: 1. neat undigested (non-matrix) 50 mM ammonium acetate solution (pH 5.5), 2. neat (non-matrix) digest samples, 3. plasma digested matrix samples and 4. urine digested matrix samples. It was observed that the MassPREP OST dTs and GalNAc prepared in the ammonium acetate exhibited better SPE recovery than the digested neat (non-matrix), plasma and urine matrix samples. Through follow-up investigation and protocol modification, using a 1:1 water dilution of the proteinase K digested samples prior to SPE loading, the MassPREP OST dTs and GalNAc oligonucleotide SPE recovery greatly improved. This 1:1 sample dilution reduced the overall salt concentration derived from both the guanidine reagent, used in the digestion, and the salts endogenously present in the urine matrix. These salts limit ion-exchange capacity of the SPE sorbent, competing with the oligonucleotide anion exchange retention on sample loading, thereby reducing SPE recovery. This capacity limitation is most pronounced when using μ Elution plate format, where sorbent bed mass/well is low (2 mg). This performance increase using the 1:1 dilution of the digested sample prior to SPE sample loading is demonstrated in Figure 3.

One of the more critical aspects was eluent composition. For a large portion of our evaluation a 50 mM TEA solution in 50% methanol routinely provided great oligonucleotide recovery >70% for the various oligonucleotides. Through continued optimization, it was discovered that an increase in TEA concentration and addition of NH₄OH solution provided a 5–10% increase in oligonucleotide recovery. For this reason, a 100 mM TEA in a 50% methanol solution containing a 0.3% NH₄OH (v:v) composition was chosen for the final OligoWorks SPE eluent. This improved performance, using 100 µL of urine sample containing the GEM91, GEM132, GalNAc, and lipid conjugated oligonucleotides digested and incubated with the RapiZyme Proteinase K module (1 hour digestion at 55 °C) with the optimized elution is highlighted in Figure 3B. Lastly an elution volume assessment was performed to leverage the key advantage of working in the OligoWorks SPE Microplate format, which is the low elution volume (25–50 µL) required for full analyte recovery, minimizing the need for sample evaporation and reconstitution. This elution study employed a 1X and 2X SPE elution step with a total of 50 µL and 100 µL elution volume, with the ultimate goal of minimizing the sample elution volume required for full elution of the oligonucleotide, and maximizing LC-MS assay sensitivity. Results of this evaluation are highlighted in Figure 3C. Comparing overall MS response, normalized to a 1 x 50 µL sample volume elution, signal for a 50 µL eluted sample was 2X that of 100 µL, indicating a 50 µL elution volume sufficiently extracted the oligonucleotide. In addition, a 2X elution strategy provided best recovery. This is expected, as there is a fundamental hold-up volume in SPE sorbent devices. For the OligoWorks SPE Microplate, the hold-up volume is ~5–7 µL. This hold-up volume contains a few % of your eluted oligonucleotide. Thus the second elution, enables full elution of the oligonucleotide from the sorbent bed, and also reduce sample variability.

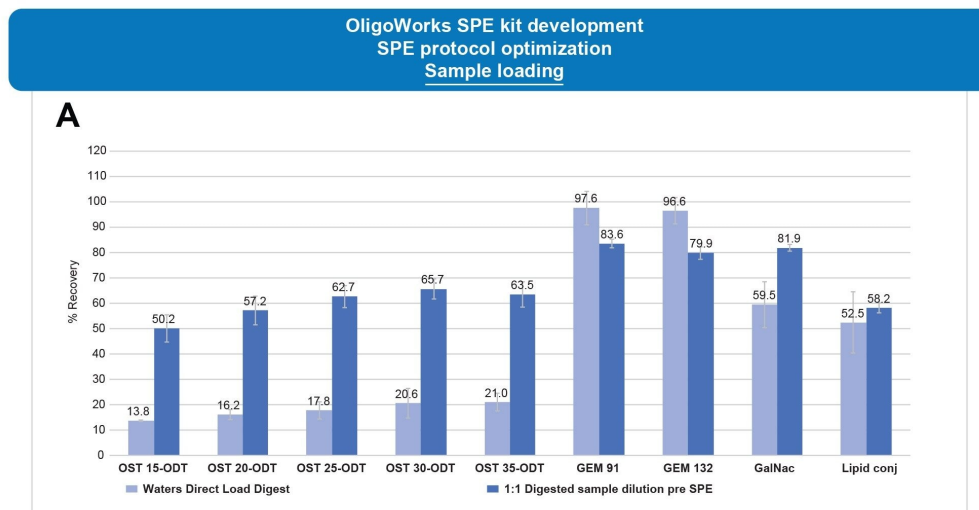


Figure 3. OligoWorks SPE Microplate Kit optimization of the digested sample loading (A), elution solution (B), and elution volume (C).

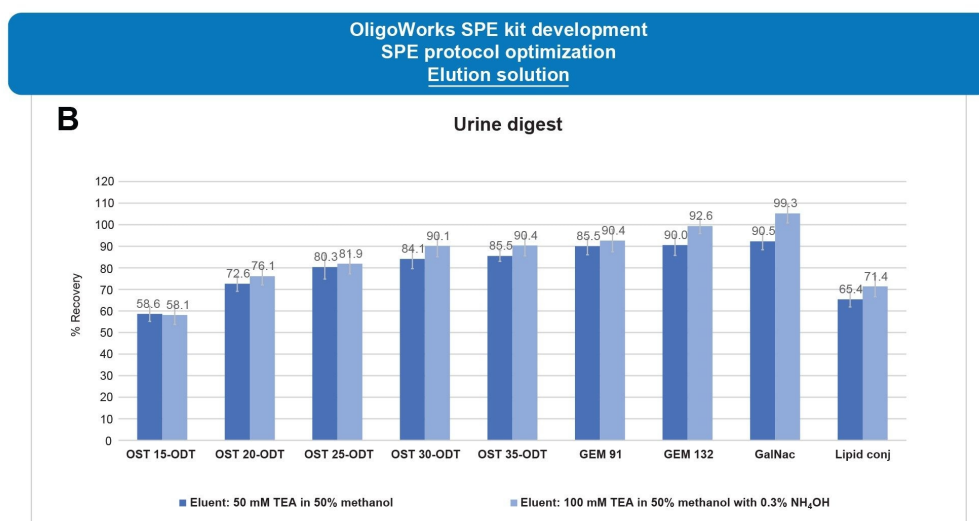


Figure 3. OligoWorks SPE Microplate Kit optimization of the digested sample loading (A), elution solution (B), and elution volume (C).

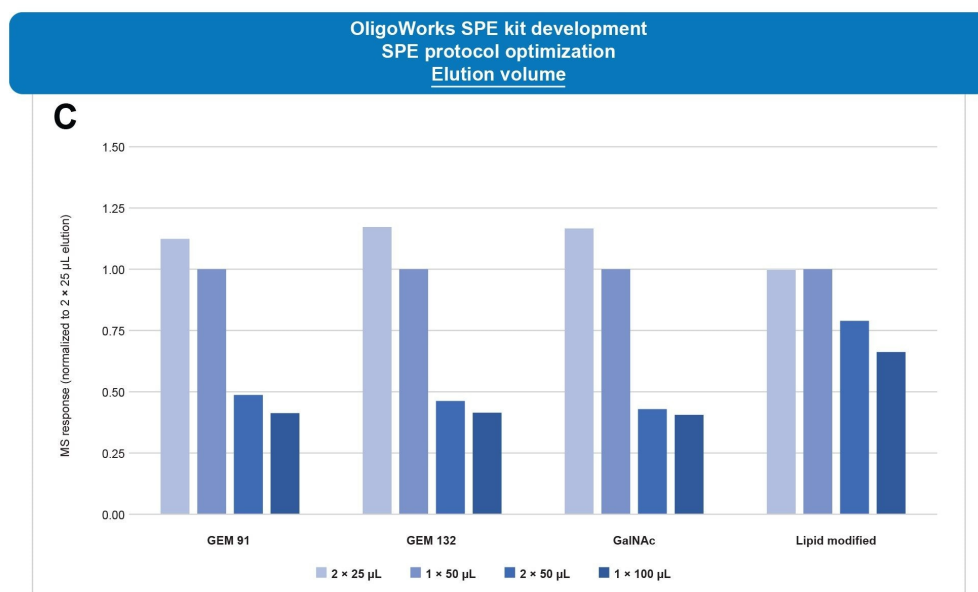


Figure 3. OligoWorks SPE Microplate Kit optimization of the digested sample loading (A), elution solution (B), and elution volume (C).

OligoWorks SPE Microplate Kit Performance

Final bioanalytical oligonucleotide extraction performance (*e.g.*, recovery, reproducibility, and matrix effects) using the OligoWorks Microplate Kit and optimized protocol was verified using the previously mentioned oligonucleotides extracted from 100 µL of urine and plasma (assessing raw area counts, with no internal standard correction) subsequently analyzed by LC-MS. Figures 4 and 5, as well as Tables 2 and 3 highlight this performance, with high oligonucleotide recovery and intra-assay reproducibility with relative standard deviations (RSDs) $\leq 15\%$, and low matrix effects (MEs). Achieving high oligonucleotide recovery improves assay limits of detection and facilitates use of low sample volumes which are common in preclinical research and discovery studies. Low matrix effects are typically an indicator of sample extract cleanliness to ensure assay specificity, while high inter/intra assay RSDs values are indicative of poor reproducibility and precision of the overall analytical assay. In addition to oligonucleotide intra-assay performance, inter-kit reproducibility was evaluated using two (2) analysts on different days, and a total of three (3) batches of RapiZyme Proteinase K Digestion Module, and six (6) batches of the OligoWorks WAX SPE Sorbent in the 96-well microplate format. For this evaluation, samples were prepared as follows, 100 µL of prepared oligonucleotide plasma samples were processed and extracted using the OligoWorks Microplate Kit and protocol with the resulting eluate analyzed by

LC-MS. Figure 6 highlights the robust and reproducible intra-kit and inter-kit oligonucleotide performance obtained from this evaluation; achieving high oligonucleotide recovery with intra-assay RSDs and batch reproducibility $\leq 5\%$ for the RapiZyme Proteinase K Digestion Module (A) and OligoWorks WAX SPE Sorbent (B). Additionally, using two (2) analysts on two (2) separate days, the calculated oligonucleotide recoveries when compared were within 15% of each other (Table 4).

Figure 7 demonstrates the OligoWorks SPE Microplate Kit flexibility using various starting plasma (A) and urine (B) volumes. Assessing raw area oligonucleotide response and using starting plasma volumes from 12.5–300 μL and urine volumes from 25–200 μL , one can see a linear increase in MS response for the GEM91 oligonucleotide, indicating that capacity has not been exceeded using the microplate SPE format. An additional advantage of the OligoWorks WAX SPE Microplate Kit and its detergent-free workflow is the direct MS compatibility of the extracted samples with as little as 25 μL elution volume. This allows for greater sample concentration, while eliminating the need for laborious evaporation and reconstitution, and reducing potential oligonucleotide losses due to adsorption and solubilization upon reconstitution.

Demonstration of direct LC-MS compatibility of the eluate, with linear LC-MS response for the GEM 91 oligonucleotide and no peak distortion for injection volumes between 12.5–300 μL is highlighted in Figure 8, panels A and B respectively. For this analysis the ACQUITY Premier UPLC™ System, configured with a 30 μL needle and 50 μL extension loop.

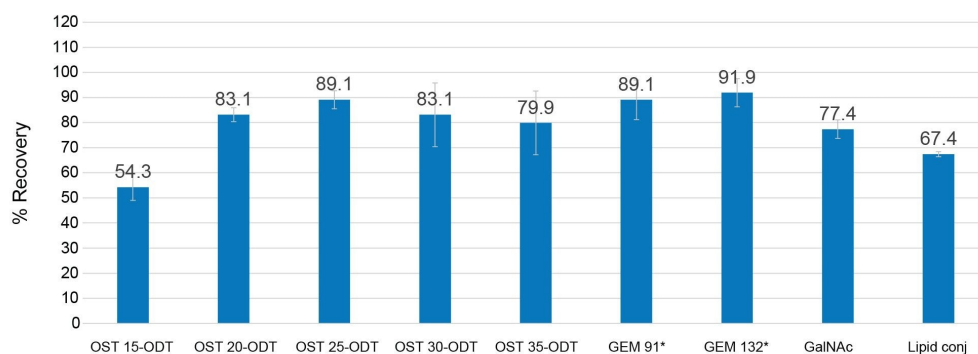


Figure 4. OligoWorks SPE Microplate Kit extraction performance (no internal standard correction) demonstrating high plasma* recoveries (1 hour digestion at 55 °C), low matrix effects, and intra-assay RSDs $\leq 15\%$ for a diversity of oligonucleotides. *A 1:1 water dilution of digested plasma sample was applied prior to SPE loading. The 1:1 dilution minimized oligonucleotide loss (break-through) on SPE sample load for the MassPREP OST and GalNAc oligonucleotides, ensuring high SPE recovery.

OligoWorks SPE Microplate Kit performance Plasma recovery, matrix effects and RSD									
Oligonucleotide	OST 15-ODT*	OST 20-ODT*	OST 25-ODT*	OST 30-ODT*	OST 35-ODT*	GEM 91	GEM 132	GalNAc*	Lipid conj
Plasma recovery	54.3	83.1	89.1	83.1	79.9	89.1	91.9	77.4	67.4
RSD	5.3	2.8	3.6	12.7	12.7	7.9	5.6	3.7	1.0
% Matrix effects	6.2	-3.5	-9.5	-10.3	7.5	6.0	-2.0	7.4	-27.3

Table 2. OligoWorks SPE Microplate Kit extraction performance (no internal standard correction) demonstrating high plasma* recoveries (1 hour digestion at 55 °C), low matrix effects, and intra-assay RSDs $\leq 15\%$ for a diversity of oligonucleotides. *A 1:1 water dilution of digested plasma sample was applied prior to SPE loading. The 1:1 dilution minimized oligonucleotide loss (break-through) on SPE sample load for the MassPREP OST and GalNAc oligonucleotides, ensuring high SPE recovery.

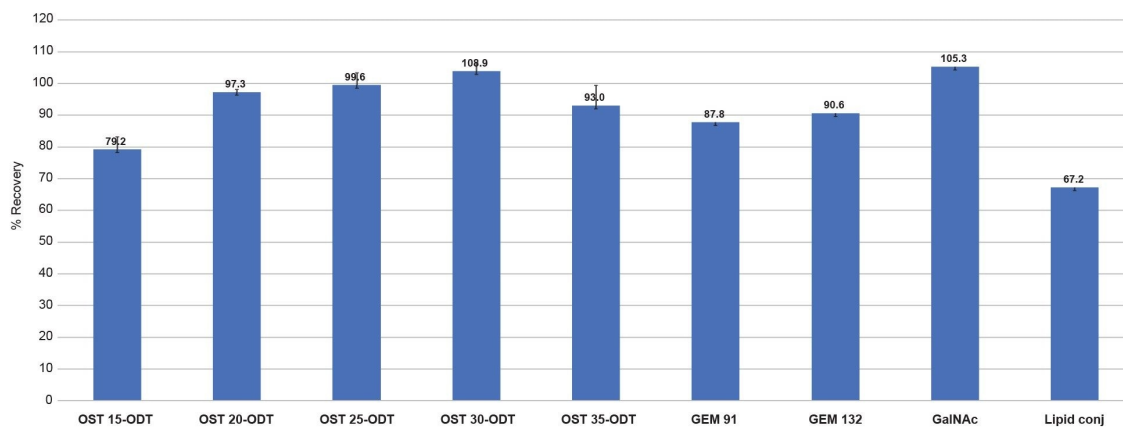


Figure 5. OligoWorks SPE Microplate Kit extraction performance (no internal standard correction) demonstrating high urine* recoveries (1 hour digestion at 55 °C), low matrix effects, and intra-assay RSDs $\leq 15\%$ for a diversity of oligonucleotides. *A 1:1 water dilution of digested urine sample was applied to all samples prior to SPE loading. This 1:1 sample dilution reduces the overall salt concentration derived from the urine matrix to minimize oligonucleotide loss (break-through) SPE sample load, ensuring high SPE recovery.

OligoWorks SPE Microplate Kit performance Urine recovery, matrix effects and RSD									
Oligonucleotide	OST 15-ODT	OST 20-ODT	OST 25-ODT	OST 30-ODT	OST 35-ODT	GEM 91*	GEM 132*	GalNAc	Lipid conj
Urine	79.2	97.3	99.6	103.9	93.0	87.8	90.6	105.3	67.2
RSD	11.5	7.4	9.8	10.1	5.8	3.6	6.2	10.6	10.4
% Matrix effects	4.2	5.2	8.4	-1.0	1.7	2.5	13.9	-11.1	-27.3

Table 3. OligoWorks SPE Microplate Kit extraction performance (no internal standard correction) demonstrating high urine* recoveries (1 hour digestion at 55 °C), low matrix effects, and intra-assay RSDs $\leq 15\%$ for a diversity of oligonucleotides. *A 1:1 water dilution of digested urine sample was applied to all samples prior to SPE loading. This 1:1 sample dilution reduces the overall salt concentration derived from the urine matrix to minimize oligonucleotide loss (break-through) SPE sample load, ensuring high SPE recovery.

OligoWorks SPE Microplate Kit performance
batch-to-batch reproducibility OligoWorks RapiZyme proteinase K digestion module
oligonucleotide plasma recovery $\leq 15\%$ SD across batches

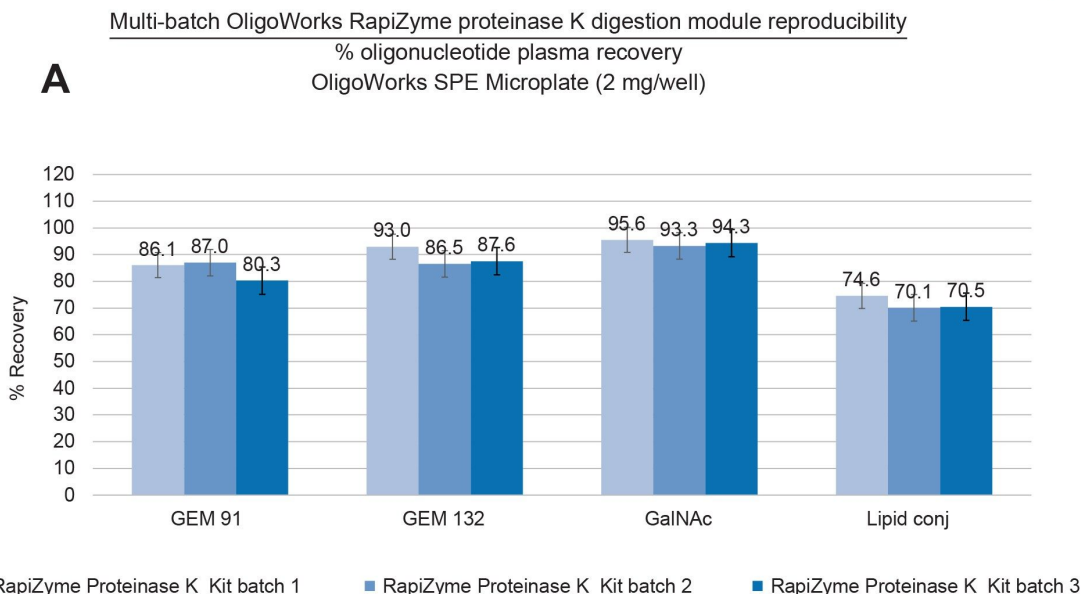


Figure 6. OligoWorks Kit SPE Microplate Kit inter-batch performance, demonstrating high plasma recovery performance (no internal standard correction) and inter and intra-assay RSDs $\leq 15\%$ for a diversity of oligonucleotide therapeutics extracted from 100 μL starting plasma volumes using the supplied starting protocol (1 hour digestion at 55°C).

for 3 batches of RapiZyme Proteinase K Digestion Module (A) and 6 batches of OligoWorks WAX SPE Sorbent (B) in the 96-well microplate format.

OligoWorks SPE Microplate Kit performance batch-to-batch reproducibility $\leq 15\%$ SD
OligoWorks SPE WAX sorbent
oligonucleotide plasma recovery across batches

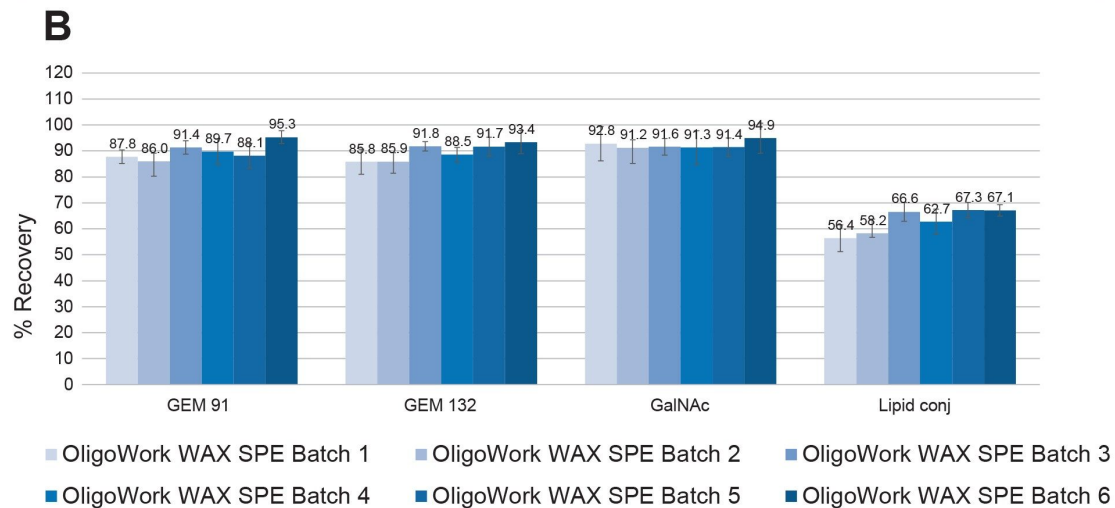


Figure 6. OligoWorks Kit SPE Microplate Kit *inter-batch* performance, demonstrating high plasma recovery performance (no internal standard correction) and inter and intra-assay RSDs $\leq 15\%$ for a diversity of oligonucleotide therapeutics extracted from 100 μL starting plasma volumes using the supplied starting protocol (1 hour digestion at 55°C).

for 3 batches of RapiZyme Proteinase K Digestion Module (A) and 6 batches of OligoWorks WAX SPE Sorbent (B) in the 96-well microplate format.

OligoWorks SPE Microplate Kit performance									
User-to-user and day-to-day									
oligonucleotide plasma recovery reproducibility $\leq 15\%$ across users and days									
OligoWorks user-to-user reproducibility: plasma recovery difference									
	OST 15-ODT	OST 20-ODT	OST 25-ODT	OST 30-ODT	OST 35-ODT	GEM 91*	GEM 132*	GalNAc	Lipid conj
User-user day 1	1.7	0.1	6.5	-0.6	7.7	5.3	4.0	-0.2	12.6
User-user day 2	3.0	7.4	13.6	-5.5	1.8	1.8	-0.2	-1.9	4.0
OligoWorks day-to-day reproducibility: plasma recovery difference									
Day-to-day user 1	1.6	-3.8	-0.3	-8.4	8.3	0.4	-5.2	-3.6	-15.3
Day-to-day user 1	2.9	3.4	6.8	-13.3	2.3	-3.0	-9.4	-5.2	-23.9

Table 4. Inter-day/inter-user OligoWorks Kit performance (no internal standard correction) demonstrating $\leq 15\%$ difference in oligonucleotide plasma recoveries ($N=4$) from 100 μL starting sample volume across 2 users and 2 days using the standard starting protocol (1 hour digestion at 55 $^{\circ}\text{C}$).

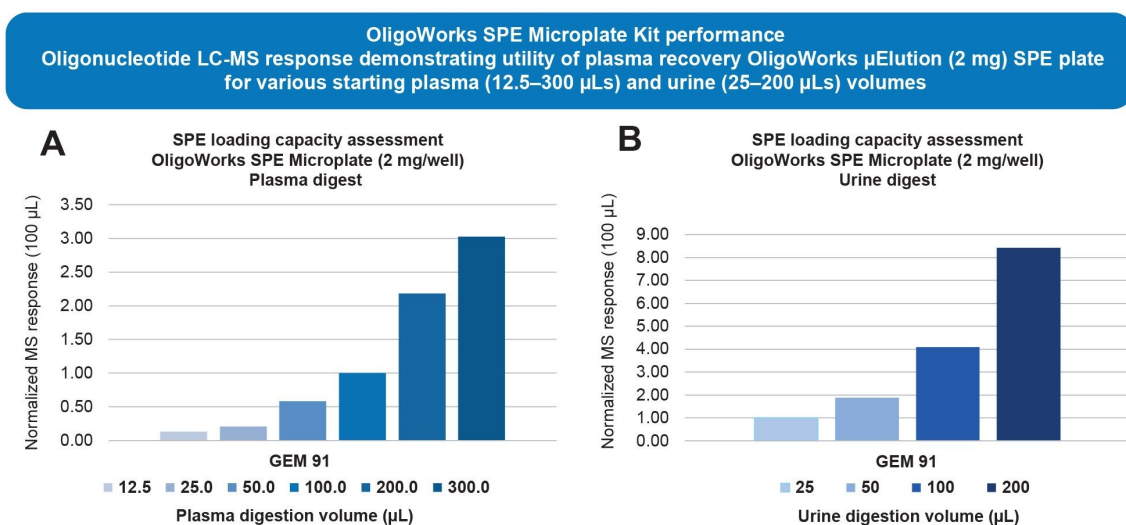


Figure 7. Demonstration of OligoWorks WAX SPE Microplate Kit flexibility, with linear LC-MS response for the GEM91 oligonucleotide plasma volumes 12.5–300 μL (A) and urine volumes 25–200 μL (B), using the OligoWorks protocol (1 hour digestion at 55 $^{\circ}\text{C}$).

OligoWorks SPE Microplate Kit performance
SPE eluent direct LC-MS compatibility
Linear oligonucleotide MS response 1–30 μ Ls

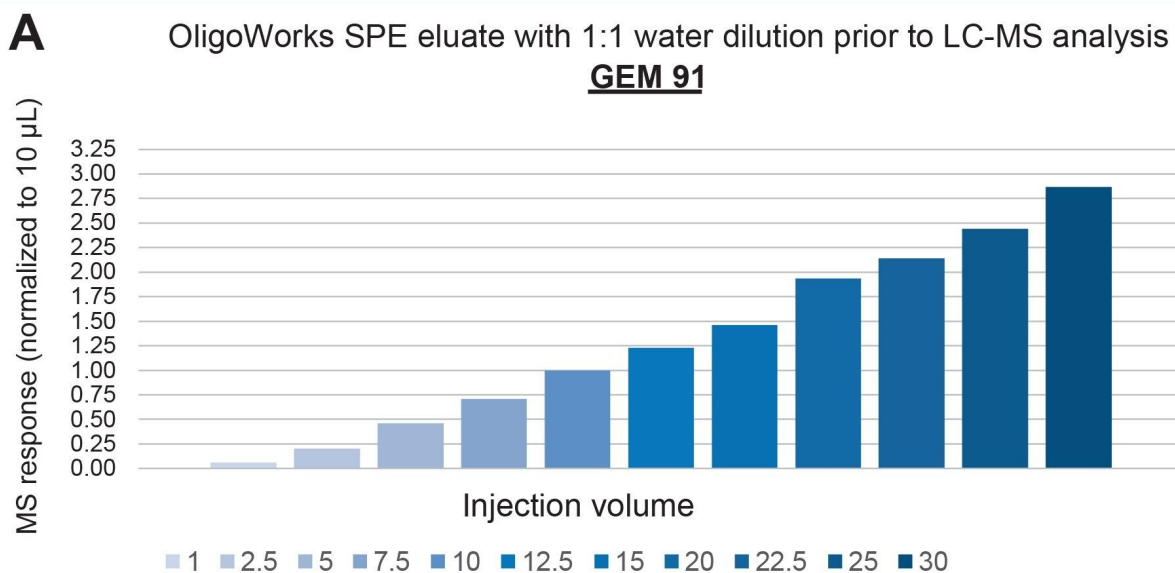


Figure 8. Demonstration of direct LC-MS compatibility of the eluate, with linear LC-MS response for the GEM 91 oligonucleotide (A), no peak distortion for injection volumes between 12.5–300 μ L (B). For this analysis the ACQUITY Premier UPLC™ System, configured with a 30 μ L needle and 50 μ L extension loop.

OligoWorks SPE Microplate Kit performance
SPE eluent direct LC-MS compatibility
Linear oligonucleotide MS response 1–30 μ Ls

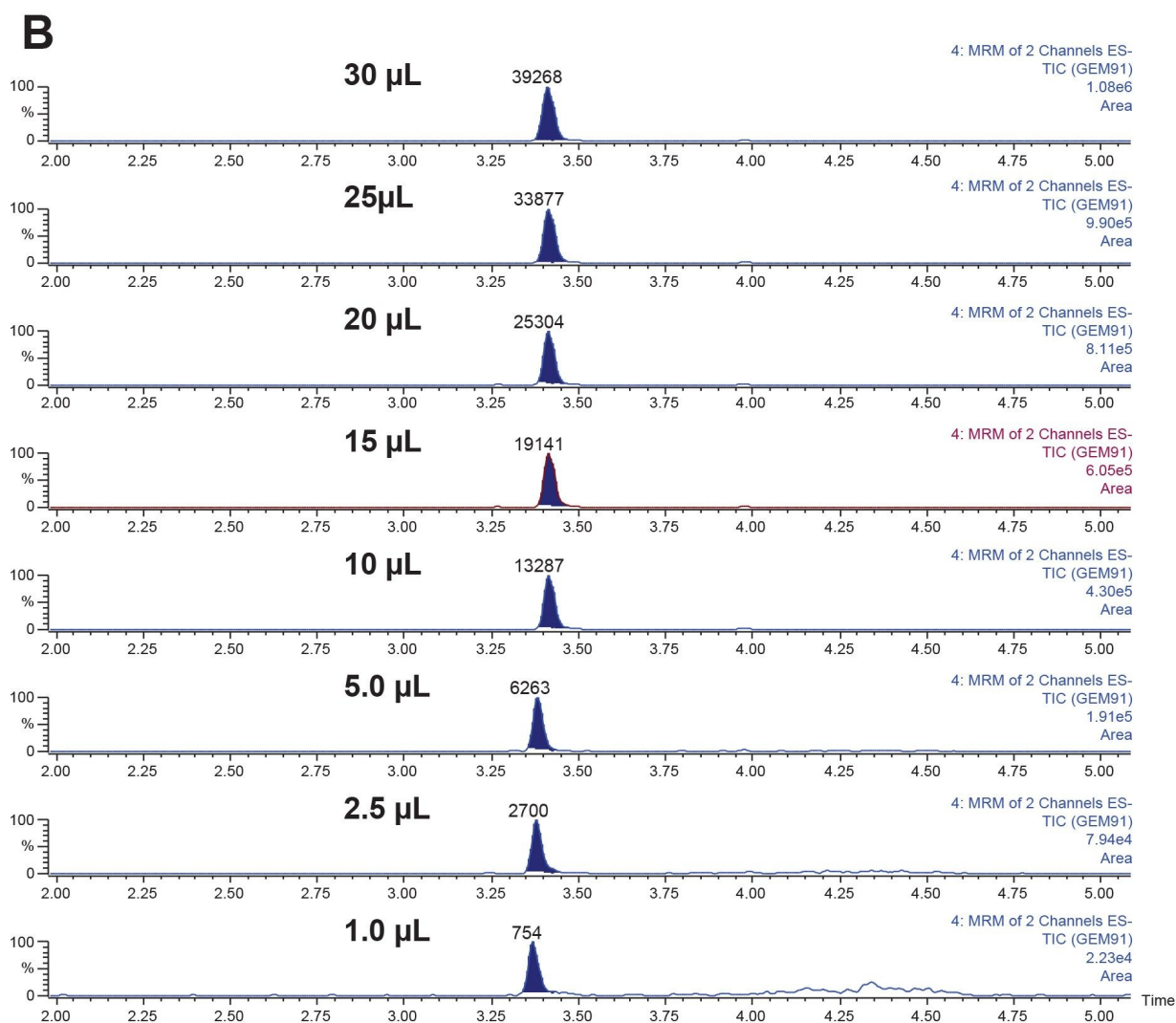


Figure 8. Demonstration of direct LC-MS compatibility of the eluate, with linear LC-MS response for the GEM 91 oligonucleotide (A), no peak distortion for injection volumes between 12.5–300 μ L (B). For this analysis the ACQUITY Premier UPLC™ System, configured with a 30 μ L needle and 50 μ L extension loop.

Conclusion

This application successfully highlights the analytical performance of the OligoWorks SPE Microplate Kit with high plasma and urine recovery (>80%), with excellent inter and intra-kit, day-to-day and user-to-user reproducibility (RSDs $\leq 15\%$) for a diverse range of oligonucleotide therapeutics, including unmodified, highly modified, and both GalNAc and Lipid conjugated modalities.

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

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[ACQUITY Premier System <https://www.waters.com/waters/nav.htm?cid=135077739>](https://www.waters.com/waters/nav.htm?cid=135077739)

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