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# Quantification of Oligonucleotide Therapeutics in Plasma Using a Generic Kit-Based Approach

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## PURPOSE

Developing LC-MS oligonucleotide bioanalytical sample preparation workflows are complex and laborious with no universal extraction method, often requiring skilled scientists and long method development times to achieve high extraction efficiency for highly sensitive, accurate and robust assays. This work leverages a generic kit-based approach with automated sample preparation and extraction to quantify multiple oligonucleotide therapeutics from plasma biomatrices, with minimal to no sample extraction method development while achieving high oligonucleotide recoveries (>75%), with excellent batch, day and user reproducibility.

## OBJECTIVE(S)

- Highlight a standardized, streamlined approach to oligonucleotide bioanalytical sample preparation using a prototype sample preparation and extraction kit.
- Demonstrate accurate and reproducible bioanalytical quantification of therapeutic oligonucleotides using the Prototype AX-SPE Microplate Kit automated on the Andrew+™ Pipetting Robot.

## METHOD(S)

### Oligonucleotides

1. Oligonucleotide deoxythymidine nucleotide (dT) mix, containing a mix of 15-35 nucleotides, Waters Milford Mass (P/N 186004135).
2. Gene expression modulators (GEM91 and GEM132), a 25-mer phosphorothioated antisense oligonucleotide and 20-mer phosphorothioated antisense oligonucleotide with 2’ methoxy caps (Nitto Denko Avecia, Milford, MA)
3. N-Acetylgalactoseamine (GalNAc) conjugated -siRNA oligonucleotide (Gifted by Alnylam Pharmaceutical)
4. Single-stranded DNA (ssDNA), 20-mer oligonucleotide, Waters Milford Mass (P/N 186009451)
5. A lipid-conjugated 16-mer antisense oligonucleotide with 5’ palmitate modification, a phosphorothioated backbone and terminal methoxy ethyl modifications (BioSearch Technologies, Lystrup, Denmark)

### LC-MS Analysis

LC-MS/MS analysis was performed using a Waters Xevo® TQ-XS tandem quadrupole MS (ESI-) and chromatographic separation using an ACQUITY I-Class PLUS UPLC® system and ACQUITY PREMIER Oligonucleotide BEH $C_{18}$, 1.7 µm, 2.1 x 50 mm column (Waters, P/N 186009452). A flow rate of 0.6 mL/min and shallow gradient was employed using 1% HFIP (Hexafluoro-2-propanol) 0.1% DIPEA (N,N-Diisopropylethylamine) in $H_2O$ and acetonitrile . Total analysis time was 5 minutes. Injection volumes of the extracted sample were 1-30 µL..

### Sample Preparation and Extraction

Oligonucleotide concentrations in plasma were between 0.025-1, µg/mL and/or 0.01-1 pmol/µL. Starting sample volumes used for Prototype AX- SPE Microplate Kit development and evaluation were between 12.5-300 µL, with 100 µL being the optimal starting volume.

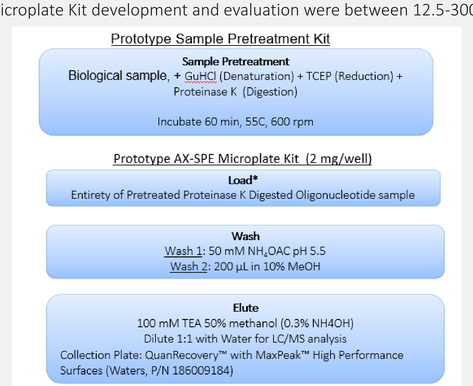


**Figure 1. Graphical representation of the oligonucleotide sample protocol using the oligonucleotide Prototype AX-SPE Microplate Kit.**

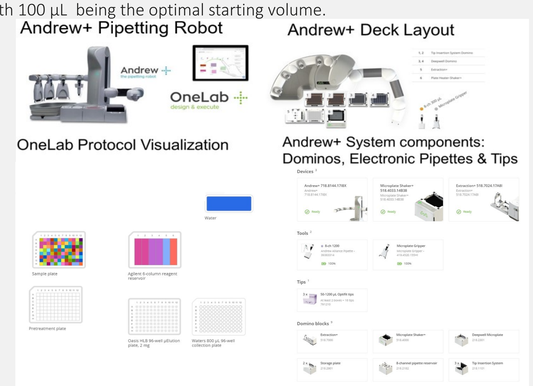


**Figure 2. Andrew+ Pipetting Robot, its OneLab deck Layout, dominos and pipettes used for plasma sample preparation extraction using the Prototype AX-SPE Microplate kit.**

## RESULT(S)

| 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 | 83.3 | 89.0 | 93.6 | 88.7 | 87.7 | 86.1 | 93.0 | 95.6 | 74.6 |
| RSD | 6.2 | 1.9 | 4.5 | 8.9 | 6.7 | 5.4 | 4.1 | 4.9 | 4.8 |
| % Matrix Effects | -0.7 | -14.5 | -4 | -12.6 | 1.4 | -0.4 | -10.3 | -6.2 | -35.2 |

**Table 1. Prototype AX-SPE Microplate Kit extraction performance (no internal standard correction) demonstrating high plasma recovery (1 hour digestion @55°), low matrix effects, and intra-assay RSDs ≤15 % for a diversity of oligonucleotides.**

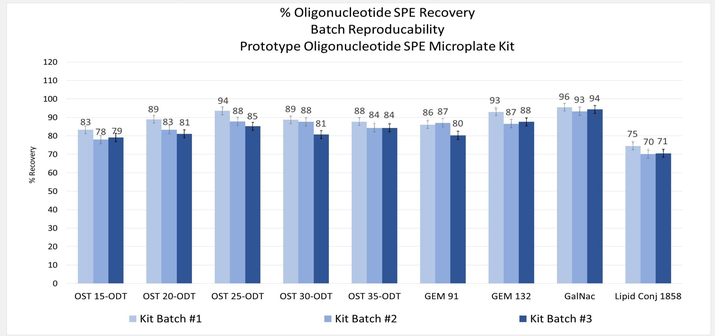


**Figure 3. Prototype AX-SPE Microplate Kit extraction performance (no internal standard correction) demonstrating high plasma recovery (1 hour digestion @55°), and inter-batch and intra-assay RSDs ≤15 % for a diversity of oligonucleotides.**

| Oligonucleotide Prototype Sample Prep Kit Reproducibility | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| User-to-User % Plasma Recovery Variation | | | | | | | | | |
| | 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 |
| Day-to-Day % Plasma Recovery Variation | | | | | | | | | |
| 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 2 | 2.9 | 3.4 | 6.8 | -13.3 | 2.3 | -3.0 | -9.4 | -5.2 | -23.9 |

**Table 2. Inter-day/inter-user Prototype AX-SPE Microplate Kit extraction performance (no internal standard correction) demonstrating ≤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 @55°).**

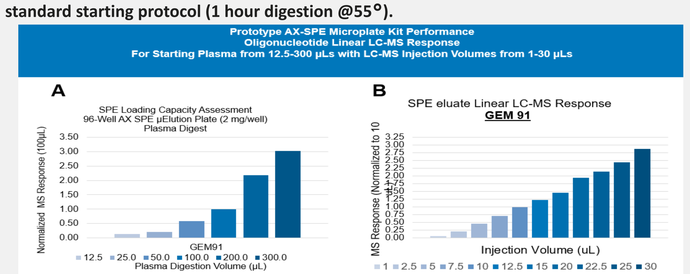


**Figure 4. Prototype AX-SPE Microplate Kit flexibility demonstrating linear LC-MS response for the GEM91 with extracted plasma volumes 12.5-300 µL (A) and for LC injection volumes from 1-30 µLs (B).**

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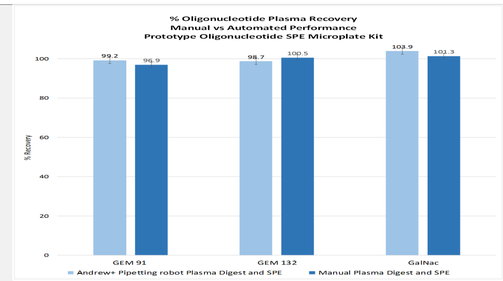


**Figure 5. Prototype AX-SPE Microplate Kit Andrew+ Pipetting Robot automated extraction performance (no internal standard correction) demonstrating high oligonucleotide plasma recovery, reproducibility, and comparability to manual preparation (1 hour digestion @55°).**

**Sensitive, Linear, Accurate & Precise**

| Calibration Curve Statistics | | | | | |
|---|---|---|---|---|---|
| Analyte | Range | Weighting | Linear Regression | % Accuracy Range | % CV Range |
| GEM91 | 0.250-1000 ng/mL | 1/x | >0.99 | 85.4-114.7 | 2.01-11.4 |
| GalNAc | | | | 85.2-114.4 | 2.01-13.44 |
| GEM132 | | | | 85.9-119.2* | 1.97-9.67 |
| ss DNA (20-mer) | 0.5-1000 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 | 99.8 | 6.77 |
| ss DNA (20-mer) | | | 763.63 | 101.8 | 4.66 |

**Figure 6. Prototype AX-SPE Microplate Kit Andrew+ Pipetting Robot automated extraction performance (no internal standard correction) demonstrating linear, accurate and precise quantitation.**

## CONCLUSION(S)

This application successfully highlights a standardized, streamlined kit-based approach to oligonucleotide bioanalytical sample preparation. Using the Prototype AX-SPE Microplate Kit with optimized protocol, and pre-measured, QC-verified, lot-traceable detergent-free reagents, high analytical performance was achieved. Percent plasma recoveries of > 75%, with excellent inter and intra-kit, day-to-day and user-to-user reproducibility (RSDs ≤ 15%) for a diverse range of plasma volumes and oligonucleotide therapeutics and including unmodified, highly modified and both GalNAc and Lipid conjugated modalities was achieved. Automated sample preparation with Andrew+ Pipetting Robot, ensured linear, accurate, robust and reproducible quantitation performance with % accuracies within 15% of expected values and RSDs ≤15 %.

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