Antisense Oligonucleotide Extraction Optimization Reveals Significant Differences Between Non-Human Primate Spinal Cord, Mouse Spinal Cord, and Mouse Brain Tissue Homogenates

Abstract Number: 583

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The authors declare no competing financial interests.

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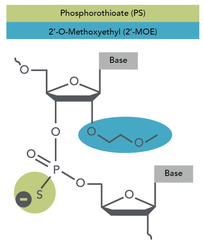
Introduction

Chemical modifications made to oligonucleotides can assist in absorption, distribution, and metabolism. While many oligonucleotide-based drugs share similarities in extraction and ionization techniques, the properties of the matrix itself are often overlooked. Here we show the extraction methods for a novel antisense oligonucleotide (ASO) in spinal cord and brain tissues vary significantly. After inconsistent results using a Clarity OTX Solid Phase Extraction (SPE) plate, the extraction in mouse brain tissue was optimized using a liquid-liquid method involving phenol-chloroform. Optimization in spinal cord tissue, however, required a more robust method using a phenyl solid-phase extraction (SPE) plate, triethylammonium acetate buffer (TEAA), and a protein precipitation filter plate. Furthermore, different tissues within the same species (mouse) required different extraction methods.

Methods

A 25.0 uL aliquot of the ASO spiked in human plasma K2EDTA containing either non-human primate spinal cord, mouse spinal cord, or mouse brain tissue was combined with 50.0 uL of internal standard (Waters MassPREP OST). From here, the brain homogenate was extracted using concentrated ammonium hydroxide, phenol:chloroform:isoamyl alcohol, water, and dichloromethane to separate the aqueous and organic layers. Mouse and cynomolgus monkey spinal cord were combined with TEAA and pulled through a phenyl SPE plate (Biotage). After eluting with 1% N,N-Diisopropylethylamine in methanol, the samples were pulled through a protein precipitation filter plate (Phenomenex). For all method types, after dry-down and reconstitution, the analyte was quantified using a Q-Exactive (Thermo) in PRM mode and a DNAPac RP column (Thermo).

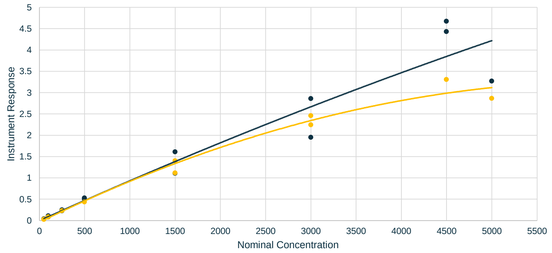
Compound



17mer Oligonucleotide

Method Development

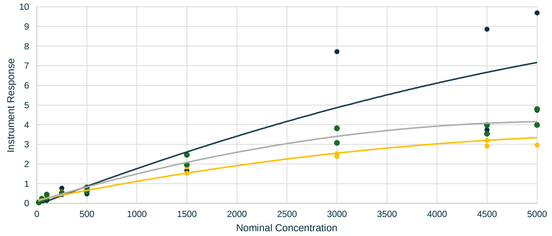
During previous developments of High-Resolution LC-MS methods for this ASO in non-human primate cerebral spinal fluid and brain homogenate, we observed that the Clarity OTX SPE plate performed adequately and produced an acceptable quadratic regression between 20.0 - 5,000 ng/mL.



NHP-CSF	
R^2	0.9678
%CV	9.9

NHP-Brain	
R^2	0.9689
%CV	7.8

We obtained inconsistent results upon further development of this analyte in new matrices, namely mouse brain, mouse spinal cord, and non-human primate spinal cord homogenate. Some of the main challenges included significant divergence above 3,000 ng/mL, two separate calibration curve slopes, and a non-linear (quadratic) response.



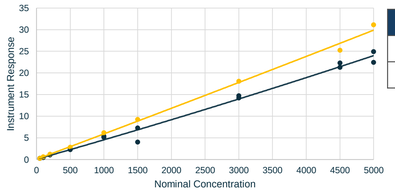
NHP-Spinal Cord	
R^2	0.8416
%CV	22.9

Ms-Spinal Cord	
R^2	0.7550
%CV	31.4

Ms-Brain	
R^2	0.6347
%CV	43.9

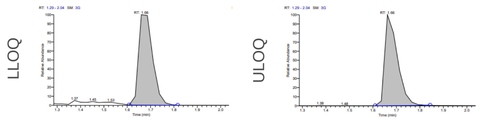
Method Development (continued)

Extraction technique optimization included separating the analyte from the aqueous phase using phenol-chloroform-isoamyl-alcohol, water, and dichloromethane. This separation technique could lead to particulates from the homogenate to be disassociated from the ASO to prevent any disruption in signal during instrumentation and analysis. From these experiments, we observed that mouse brain homogenate performed within our acceptance criteria. The instrument response ratio showed a quadratic regression yet there were no divergent curves nor two slopes in the calibration curve.

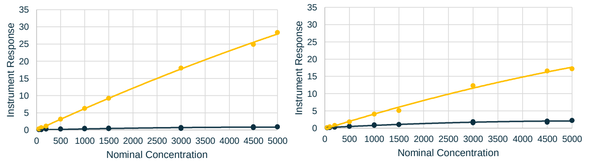


Ms-Brain	
R^2	0.9930
%CV	5.3

Plasma Only Curve | Tissue + Plasma Curve



Interestingly, when the ASO was extracted without tissue homogenate (plasma only), a higher instrument response ratio was quantified (10- to 30-fold increase across the calibration curve) when compared to samples with tissue homogenate and plasma combined.



NHP-Spinal Cord	
R^2	0.8416
%CV	22.9

Plasma Only Curve | Tissue + Plasma Curve

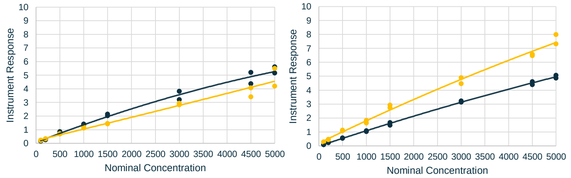
Ms-Spinal Cord	
R^2	0.5239
%CV	71.7

Plasma Only Curve | Tissue + Plasma Curve

Nonetheless, spinal cord homogenate in both species continued to show divergence and two calibration curve slopes with this technique. Prior to further experimental design optimization, instrumentation testing showed no suppression was impacting the samples during the retention times of interest. Additionally, plasma-only samples spiked with tissue showed similar results, indicating a true matrix effect for each tissue.

Method Development (continued)

As an alternative extraction technique, we turned to a phenyl SPE plate to clean up the homogenized samples and prevent major matrix effects. Loading the ASO onto the plate with TEAA and eluting with 1% DIPEA in (water/MeOH 10/90 v/v) then passing through a filter plate prior to injection showed a significantly improved linearity within the calibration curve, no divergence, minimal response %CV values, and acceptable results using both linear and quadratic regressions.

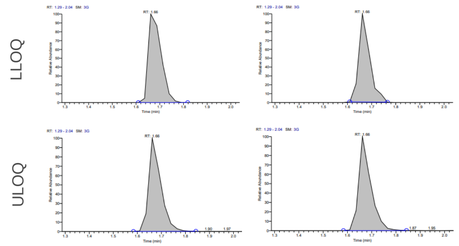


NHP-Spinal Cord	
R^2	0.9874
%CV	8.3

Plasma Only Curve | Tissue + Plasma Curve

Ms-Spinal Cord	
R^2	0.9912
%CV	6.4

Plasma Only Curve | Tissue + Plasma Curve



Conclusion

An ASO in spinal cord and brain tissues requires individualized and significantly different DNA-based extraction methods for optimal, reproducible results. Sample analysis with these methods yielded 100% acceptance rates (data below).

NHP-Spinal Cord	
R^2	0.9860
%CV	6.6

Ms-Spinal Cord	
R^2	0.9905
%CV	6.6

Ms-Brain	
R^2	0.9903
%CV	6.8