M1130-06-31

Phenyl SPE: A Promising Alternative to More Common Oligonucleotide Extraction

Davies BM[1], Wilcock B[1], Fang T[2], Bettencourt B[2], Hardcastle C[1], Voelker T[1]

1. Aliri Bioanalysis, Salt Lake City, UT, USA
2. Third Rock NewCo, Boston, MA, USA

CONTACT INFORMATION: brandon.davies@aliribio.com

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PURPOSE

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. The extraction of a variety of oligonucleotides using a standard Clarity OTX solid phase extraction plate has been more commonplace in bioanalytical laboratories. These plates have been shown to isolate RNA and DNA-based therapeutics from biological fluids and tissues in a short period of time with high recovery percentages and standard extraction buffers. Here we show an alternate extraction method for a novel antisense oligonucleotide (ASO) in spinal cord and brain tissues. Originally, acceptable and reproducible results were obtained by using a Clarity OTX SPE plate with non-human primate cerebral spinal fluid and brain tissue homogenate. The extraction was modified in mouse brain tissue to a liquid-liquid method involving phenol-chloroform. Further 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.

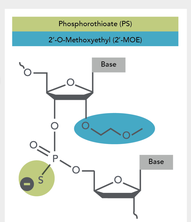
METHODS

A 25.0 µL 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 µL 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 non-human primate 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).

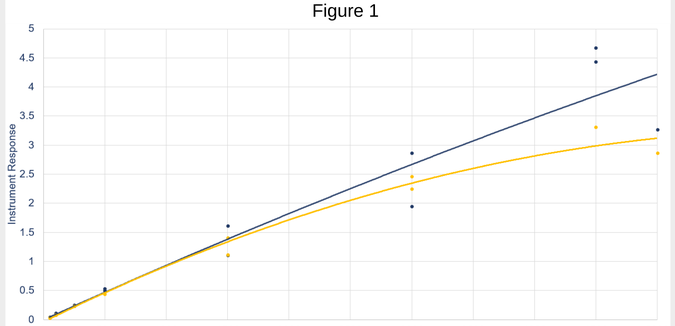
For all method types, after dry-down and reconstitution, the analyte was quantified using a Q-Exactive (Thermo) in parallel reaction monitoring (PRM) mode and a DNAPac RP column (2.1 x 50 mm, 4 µm) (Thermo).



RESULTS

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 (Figure 1).

Figure 1

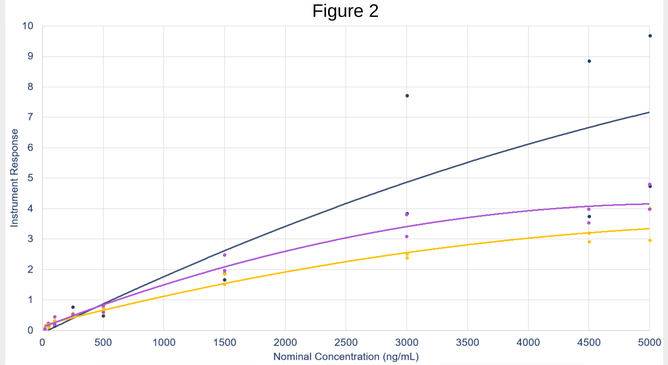


NHP-CSF	
R^2	0.9678
%CV	9.9

NHP-Brain	
R^2	0.9689
%CV	7.8

Upon further development of this compound in new matrices, namely mouse brain, mouse spinal cord, and non-human primate spinal cord homogenate, we obtained inconsistent results (Figure 2). Some of the main challenges included significant divergence above 3,000 ng/mL, two separate calibration curve slopes, and a non-linear (quadratic) regression.

Figure 2



NHP-Spinal Cord	
R^2	0.8416
%CV	22.9

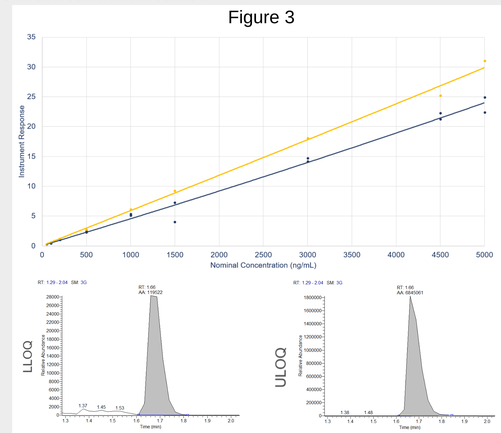
Ms-Spinal Cord	
R^2	0.7550
%CV	31.4

Ms-Brain	
R^2	0.6347
%CV	43.9

RESULTS (CONTINUED)

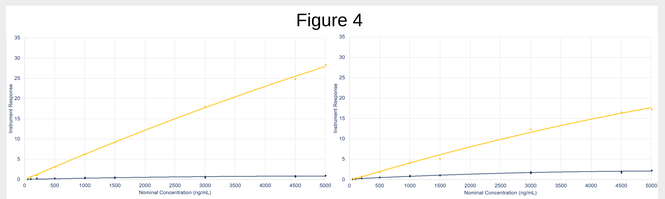
Extraction technique optimization included separating the analyte from the aqueous phase using phenol-chloroform-isoamyl-alcohol, water, and dichloromethane. This separation technique could lead to the dissociation of particulates from the ASO in the homogenate to prevent any disruption in signal during instrumentation and analysis. From these experiments, we observed that mouse brain homogenate performed within our acceptance criteria (Figure 3). The instrument response ratio showed a linear regression with no divergent curves nor two slopes in the calibration curve.

Figure 3



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 (Figure 4).

Figure 4



NHP-Spinal Cord		Plasma Only Curve	Tissue + Plasma Curve
R^2	0.8258		
%CV	32.0		

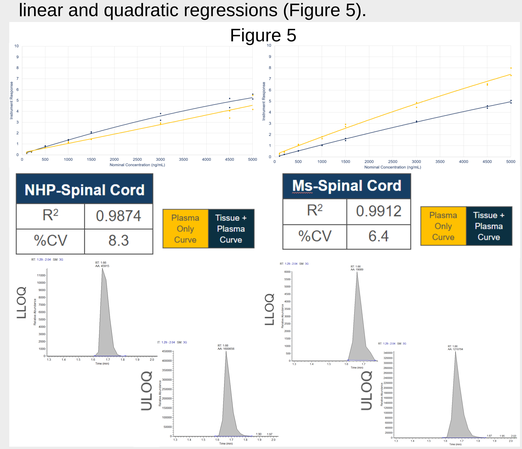
Ms-Spinal Cord		Plasma Only Curve	Tissue + Plasma Curve
R^2	0.5239		
%CV	71.7		

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 (data not shown). Additionally, plasma-only samples spiked with tissue showed similar results, indicating a true matrix effect for each tissue.

RESULTS (CONTINUED)

As an alternative extraction technique, we turned to a phenyl SPE plate to clean up the homogenized samples. 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 (Figure 5).

Figure 5



NHP-Spinal Cord		Plasma Only Curve	Tissue + Plasma Curve
R^2	0.9874		
%CV	8.3		

Ms-Spinal Cord		Plasma Only Curve	Tissue + Plasma Curve
R^2	0.9912		
%CV	6.4		

CONCLUSION

An ASO in spinal cord and brain tissues requires individualized and significantly different DNA-based extraction methods for optimal, reproducible results. This technique provides a distinct and reliable alternative to the more commonplace SPE methods when using more unique matrices. Subsequent usage of these methods in sample analysis assays produced 100% acceptable results across all matrices (see Figure 6).

Figure 6

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

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