

APPLICATIONS

Extraction of 2'-MOE Phosphorothioate from Tissue Using Clarity® OTX™ Solid Phase Extraction (SPE)

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Overview

Oligonucleotides represent an emerging therapeutic modality. Because they can modulate both gene expression and post-transcriptional splicing of introns and exons, oligos can be used to treat a wide variety of indications, including previously “undruggable targets.” However, there are many challenges associated with oligos. For analytical chemists involved in bionalytical workflows, the challenges are several fold, though particularly, the extraction of the oligo is imperative for good assay specificity, sensitivity, and linearity.

Because therapeutic oligos have unique physicochemical properties due to their myriad modifications for nuclease resistance, protein precipitation or other traditional sample preparation approaches can prove quite challenging. Further, because of their relatively low dosing (lower than 1mg/kg at times), obtaining relevant lower limits of quantitation (LLOQ) and good linearity for oligo pharmacokinetic assays is particularly challenging.

One common approach to using the novel, single step solid phase extraction (SPE) Clarity OTX. Here we present the extraction of a, 2'-methoxyethyl phosphorothioate splice switching oligo (SSO) from central nervous system (CNS) tissue.

Materials and Methods

All samples and analytes (Fully thioated, 2'-MOE Gapmer and internal standard) were purchased from Integrated DNA Technologies (Coralville, IA). All experiments were performed by a third-party contract laboratory. Analysis was performed using a Shimadzu Nexera HPLC and SCIEX Triple Quad 6500+ nominal mass spectrometer. Data processing was performed on SCIEX Analyst software. LC and MS Conditions are noted in **Tables 1 and 2**, respectively.

Sample Preparation

Sample preparation followed the sample pre-treatment, as outlined. Subsequent solid phase extraction was performed using the Clarity OTX SPE kit, 96-well plate, 100mg/well format, as per the vendor recommendations.

Sample Pre-treatment Protocol

1. Add 250 µg/mL Proteinase K in homogenization buffer
2. 2.5% IGEPAL, 5 mM EDTA, 50 mM Tris pH = 8.0, 250 µg/mL Proteinase K, 4:1 (v:w)
3. Add ceramic beads to each tube
4. Vortex using GenoGrinder or equivalent for 2 min at 1700 rpm
5. Incubate 55° C in water bath, 1 hour
6. Store homogenate at -70° C prior to use

Extraction Protocol

Aliquot 75 µL calibration standards, QC samples, study samples onto 2 mL 96-well round bottom plate (PHX Part No.: AH0-8636)

96-well Plate: Clarity OTX 100 mg/well

Part No.: 8E-S103-EGA

Condition: 1 mL MeOH

Equilibration: 1 mL Equilibration Buffer (50 mM Ammonium Acetate, pH 5.5)

Add: 600 µL of Lysis Loading Buffer to all samples

Load: Pre-treated samples after a ~5 min vortex ; incubate at RT for additional 5 minutes

Transfer: Solution to TOMTEC or equivalent

Wash 1: 3x 1 mL Equilibration Buffer

Wash 2: 3x Wash Buffer (50 mM Ammonium Acetate, pH 5.5: ACN, 50:50)

Elute: 0.5 mL Elution Buffer (100 mM Ammonium Bicarbonate, pH 9.5:ACN:THF (50:40:10, v/v/v) into the collection plate

Dry Down: to ~400 µL under a gentle stream of Nitrogen
Prior to analysis, vortex-mix samples for ~1 minute

Figure 1: Total Ion Chromatograms for QCs and Internal Standard

Total Ion Chromatogram (TIC) for QCs and Internal Standard (IST) for ASO Oligo at 1.5, 30, and 300 ng. IST returns consistent peak areas, suggesting good recoveries for each standard.

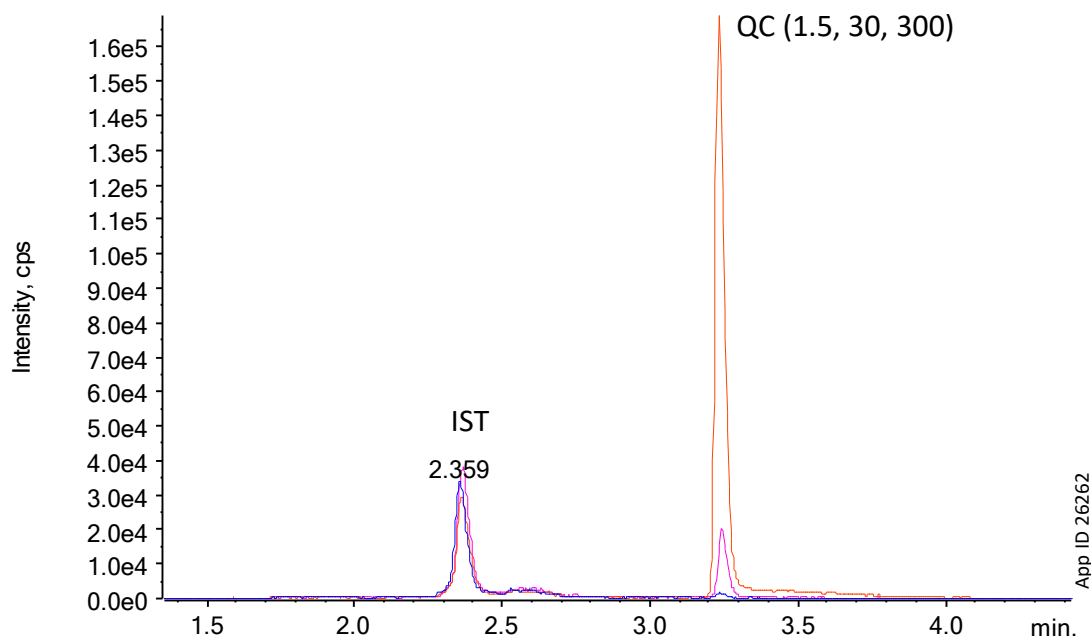


Table 1: HPLC Conditions

Column:	bioZen™ 2.6 µm Oligo
Dimension:	100 x 2.1 mm
Part No.:	00D-4790-AN
Mobile Phase A:	1.0 % HFIP, 0.1% DIPEA in Water with 10 µm EDTA
Mobile Phase B:	0.075 % HFIP, 0.0375% DIPEA in Water, Acetonitrile (35:65) with 10µm EDTA
Gradient Program:	Gradient Slope: 20-60 % B in 1.5 minutes Column Wash: 20-95%B (2x)
Flow Rate:	0.5 mL/min
Temperature:	80° C
Injection:	2 µL

Table 2: MS Conditions

Polarity:	(-)
CAD Gas:	10
Curtain Gas:	20
GS1 (psi):	50
GS2(psi):	50
Spray Voltage (V):	-4000
Temperature (C °):	500

Table 3: MRM Conditions

	Q1	Q3	Dwell	CE	DP
Sample	1017.0	393.2	50	-60	-50
IST	828.7	811.8	50	-25	-75

Table 4: Summary of Standard Curve and QCs for CNS Tissue (Brain and Spinal Cord)

ng/mL	Name	Observed	% CV	Accuracy
1	Brain Std-2	0.933	10.9	93.3
5	Brain Std-3	5.49	9.2	109.8
25	Brain Std-4	25.8	7.3	103.1
50	Brain Std-5	47.8	10.5	95.6
100	Brain Std-6	102	8.9	101.7
250	Brain Std-7	247	9.8	98.7
400	Brain Std-8	403	8.2	100.8
500	Brain Std-9	473	7.8	94.7
1.5	QC-Low Brain	1.71	15.9	114.0
1.5	QC-Low Spinal Cord	1.47	18.2	98.3
30	QC-Mid Brain	33.3	9.6	111.1
30	QC-Mid Spinal Cord	31.0	11.8	103.2
300	QC-High Brain	283	6.7	94.2
300	QC-High Spinal Cord	287	2.2	95.8

Discussion

Extraction of oligonucleotides out of various biological matrices can be challenging, but tissue presents some unique ones, including obtaining acceptable recovery and sensitivity. One potential for low recovery is sample pretreatment, i.e. the preparation of the tissue prior to homogenization and solid phase extraction. Previously reported sample pretreatment techniques^{1,2} have utilized Proteinase K, a non-specific serine protease which is commonly used for molecular biology applications requiring the homogenization of cell or tissue samples. Uniquely, Proteinase K improves under denaturing conditions, so the use of nonionic detergent was critical in the digestion. Tissue pulverization also followed the literature recommendations, with ceramic bead-based homogenization.

Following digestion and pulverization, the tissue homogenate will be more amenable to the solid phase extraction and may then be treated as any other non-solid matrix, such as serum or plasma. As demonstrated by standard curve, generated with spiked brain tissue, accuracy and percent recoveries are more than acceptable, with % CVs less than 11% for all standards and replicates.

QCs from spinal cord and brain are also good, with acceptable CVs and accuracies for low, mid and high CVs. Internal standards return consistent peak areas as well, as observed in Figure 1.

Conclusion

Bioanalytical workflows are highly dependent on sample preparation. Due to their complexity and physicochemical properties, oligonucleotides present unique challenges for extraction and clean up, especially when looking at different biological matrices, such as solid tissue.

To ensure recovery and reproducibility for oligo extraction from tissue, it is imperative that an effective sample pretreatment strategy is used. This includes a digestion with Proteinase K and tissue pulverization. Homogenate may then be treated as any other sample matrix, and extraction of the oligonucleotide may then be performed using the Clarity® OTX™ solid phase extraction platform. Optimization of the SPE itself may be necessary depending on the analyte and matrix interferences.

APPLICATIONS

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