



# Quantitative Determination of A Phosphothioate Oligonucleotide in Dog Plasma Using LC/MS/MS

## Authors

*Yue Zhao, Laixin Wang, Weiwei Yuan, Ryan Adler, Min Meng and Scott Reuschel*

## Introduction

Oligonucleotide therapeutics, including siRNA, aptamers, ribozymes and antisense oligonucleotides, are currently being investigated in the preclinical and clinical stages for various diseases. These biopharmaceuticals require robust bioanalytical assays to determine the toxicokinetic (TK)/pharmacokinetic (PK) parameters and exposure–response in order to choose the right dosage regimens of therapeutic oligonucleotides in support of their preclinical and clinical development. Hybridization based immunoassays and HPLC-UV have been predominately used for the quantitative analysis of oligonucleotides in support of TK/PK evaluations. However, neither methodology is ideal because immunoassays generally have narrow calibration ranges and poor specificity, and HPLC-UV methods typically have low sensitivity [1].

To overcome these shortcomings, we have used either UPLC-PDA [2, 3] or LC-MS/MS [4] to develop and validate dozens of assays to analyze oligonucleotides in biological matrices. However, all samples in our previously reported methods were extracted using manual liquid-liquid extractions. To improve the extraction throughput via increased automation, we have recently developed and validated an LC-MS/MS assay to analyze a proprietary 21-mer siRNA in human plasma using SPE extraction. Using a with 200  $\mu$ L plasma aliquot, the validated range for the double stranded siRNA is 25.0-5,000 ng/mL. Here we present this methodology by using a non-proprietary 18-mer phosphorothioate oligonucleotide (TL0901) extracted from dog plasma as a model compound. The dynamic range for TL0901 in dog plasma (K2EDTA) is 10.0-2,500 ng/mL.

## Methodology

### SAMPLE PREPARATION:

1. Thaw the samples at room temperature.
2. Aliquot 200  $\mu\text{L}$  of sample into the corresponding wells of a 96-well deep well plate.
3. Add 50.0  $\mu\text{L}$  of working internal standard solution [10.0  $\mu\text{g}/\text{mL}$  of TL0901 (n-6) in water].
4. Add 200  $\mu\text{L}$  of 50 mM  $\text{NaH}_2\text{PO}_4$  and 2mM  $\text{NaN}_3$  in water (pH=5.5).
5. Vortex-mix the samples thoroughly.
6. Condition a Phenomenex SPE 96-well plate with 1 mL of MeOH followed by 1 mL of 50 mM  $\text{NaH}_2\text{PO}_4$  and 2mM  $\text{NaN}_3$  in water (pH=5.5).
7. Transfer the samples onto the SPE plate using Tomtec.
8. Wash sample plate with six 1 mL volumes of 50 mM  $\text{NaH}_2\text{PO}_4$  in 50:50 Water:MeCN (v/v).
9. Elute the samples with 500  $\mu\text{L}$  of [5% TEA in 9/36/46/9 (1.0 M AmBicarb pH unadj)/water/MeCN/THF (v/v/v/v)] into a tapered 96-well plate which has been pre-filled with 50.0  $\mu\text{L}$  of DNA recovery™ solution.
10. Evaporate the samples to dryness at approximately 50 °C under nitrogen in a Turbovap.
11. Reconstitute the samples with 200  $\mu\text{L}$  mobile phase A (HFIP and TEA buffered water).

### CHROMATOGRAPHIC CONDITIONS

Column: Phenomenex C18, 2x50 mm, 3  $\mu\text{m}$

Mobile Phase: A: HFIP and TEA buffered water

B: HFIP and TEA buffered MeOH

C: 90:10 MeCN:water (backflush at 0.600 mL/min)

Gradient with column backflush

(back flush column time: 2.0' to 3.0')

Time	0.01'	2.0'	2.5'	3.5'	5.5'
B%	15	45	45	15	end

Injection volume: 5 – 20 $\mu\text{L}$

Column temperature: 55°C

Flow rate: 0.300 mL/min

AS Temperature: RT

Needle wash 1: 0.1%TEA and 0.01% EDTA in 50:50 DMF:water

Needle wash 2: Mobile phase A (HFIP and TEA buffered water)

## Methodology continued

### MASS SPECTROMETER CONDITIONS

Instrument API5000

Ionization mode: Turbo ionspray, Negative ion mode

Source Temperature: 500°C

SRM transitions:

Analyte	Internal Standard	Transitions ( $\pm 0.5$ amu)	Typical Retention Times (min.)
TL0901		715.4 $\rightarrow$ 319.0	1.5
	TL0901(n-6)	631.7 $\rightarrow$ 319.0	1.3

## Results and Discussion

### METHOD DEVELOPMENT

TL0901 is an 18-mer phosphorothiate oligonucleotide (sequence is 5'-ACTGTACG-ATTTCGACCTA) that was randomly selected by Tandem Labs for the purpose of evaluating the analytical. A 12-mer analog phosphorothiate oligonucleotide (TL0901(n-6)) was used as the internal standard.

- The molecular weight of TL0901 is 5731.6. A typical multiple-charged Q1 scan was obtained under optimized negative ESI conditions (Figure 1).
- The full length TL0901 can be completely separated chromatographically from its n-6 or shorter metabolites, but not the n-4 or longer metabolites under selected LC conditions (Figure 3). Fortunately, the analyte and each metabolite have multiple MRM transitions to choose to avoid interfering with each other (Figure 1, 2 and 4).
- The extraction recoveries of the SPE method in this report are comparable to the previous LLE method [4] for TL0901 and its metabolites (~80%). However, the extraction recoveries significantly decrease as the length of the phosphorothiate oligonucleotide increases.

## Results and Discussion continued

### METHOD QUALIFICATION

A full accuracy and precision batch was extracted and analyzed with duplicate calibration curves and 5 levels of quality controls (QC) at n=6 each. Both the linearity (Figure 4) and accuracy/precision obtained for the method (Table 1) were excellent.

FIGURE 1. Typical Q1 Scan Mass Spectrum of TL0901.

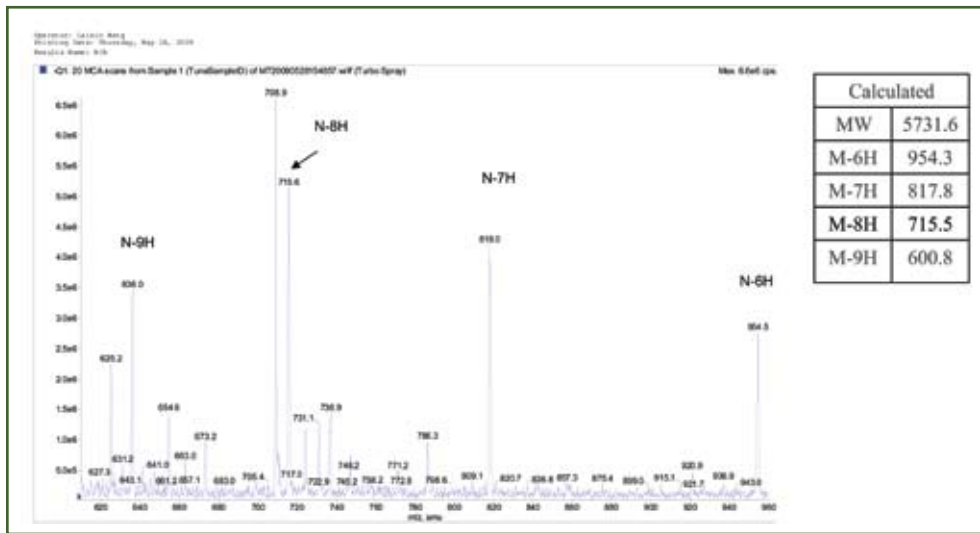
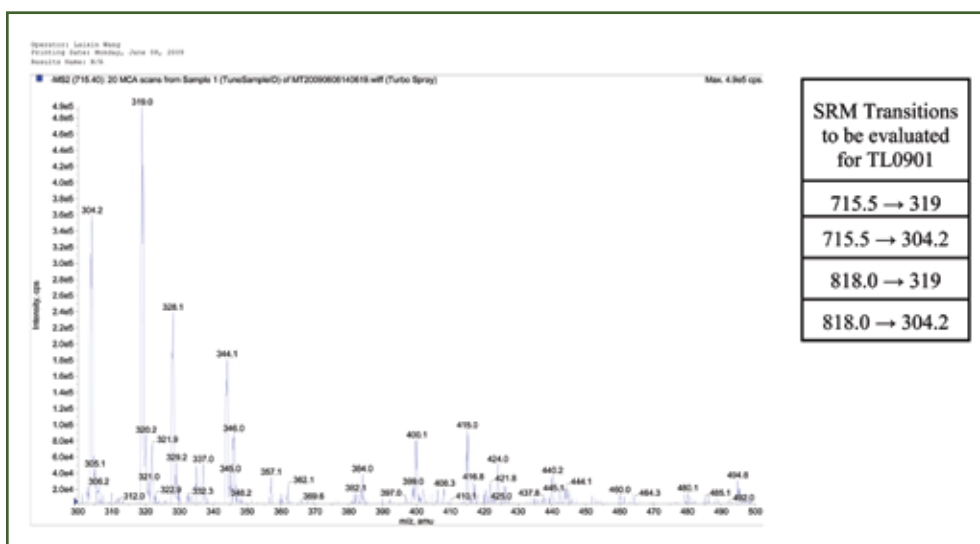


FIGURE 2. Typical Product Ion Scan Spectrum of TL0901





## Results and Discussion continued

FIGURE 3. Liquid Chromatographic Separation of TL0901 and Its Metabolites

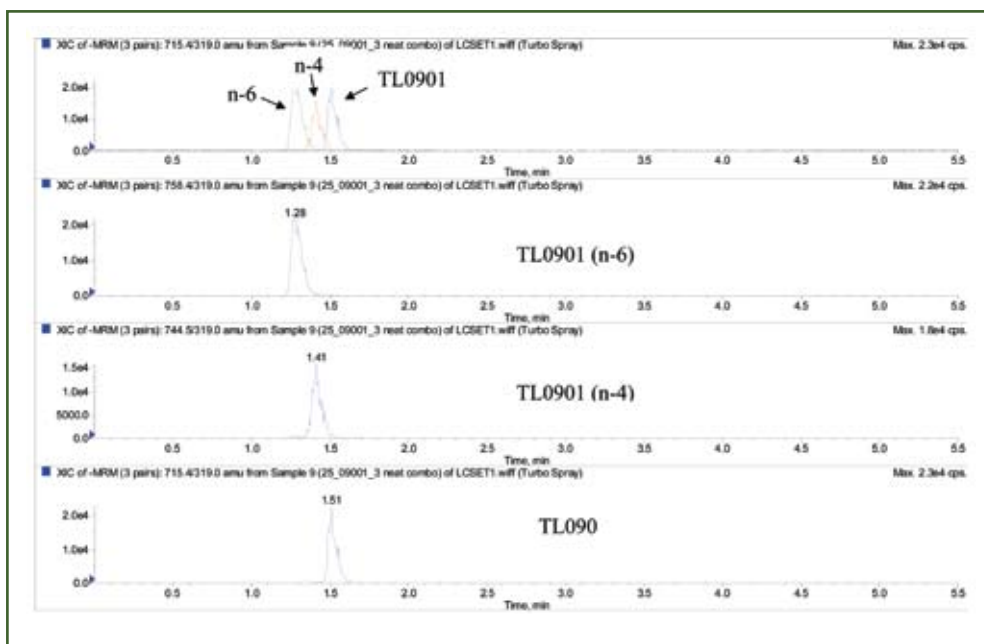
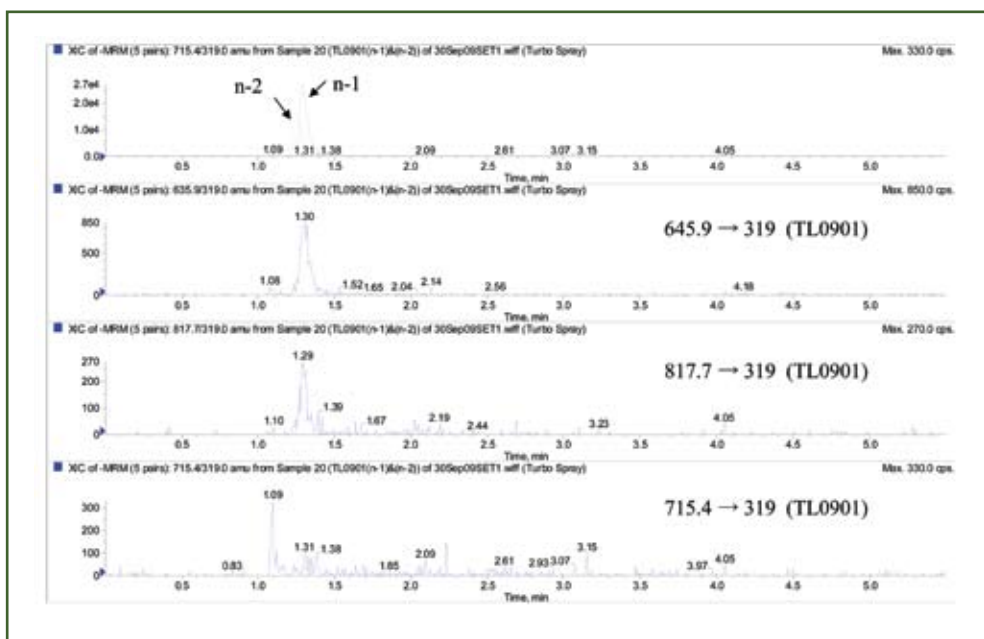


FIGURE 4. MS/MS Selectivity and LC Separation of TL0901 and Its n-1 and n-2 Metabolites







## Results and Discussion continued

FIGURE 7. Representative Blank with Internal Standard (0-ng/mL QC)

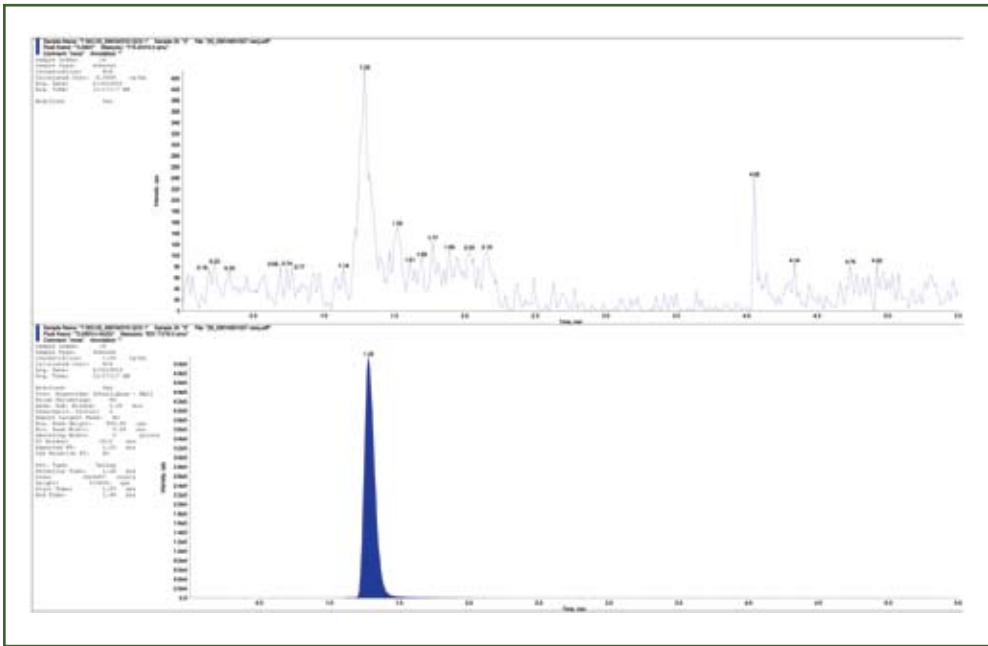
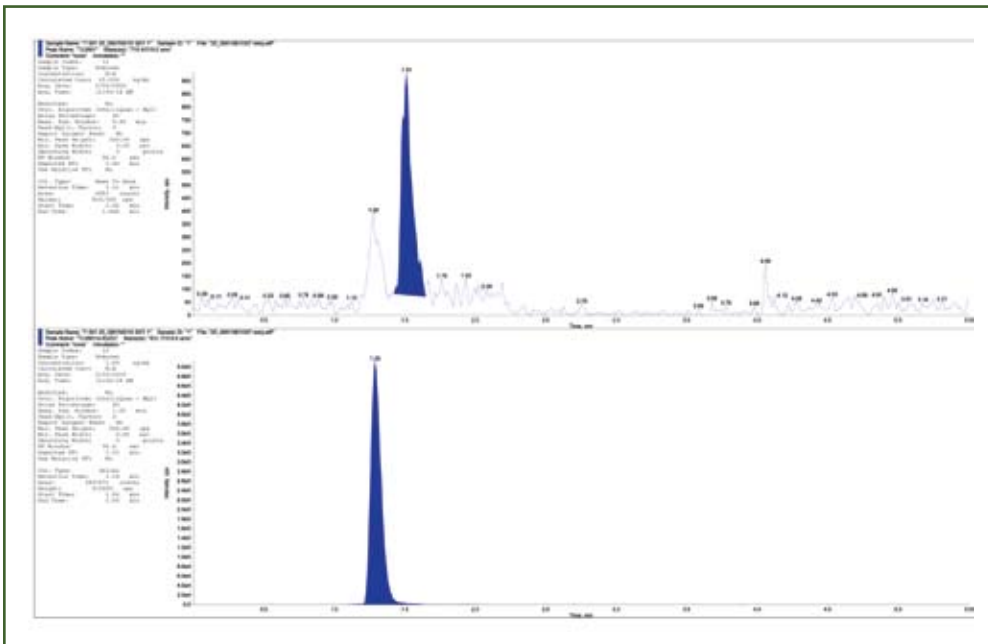
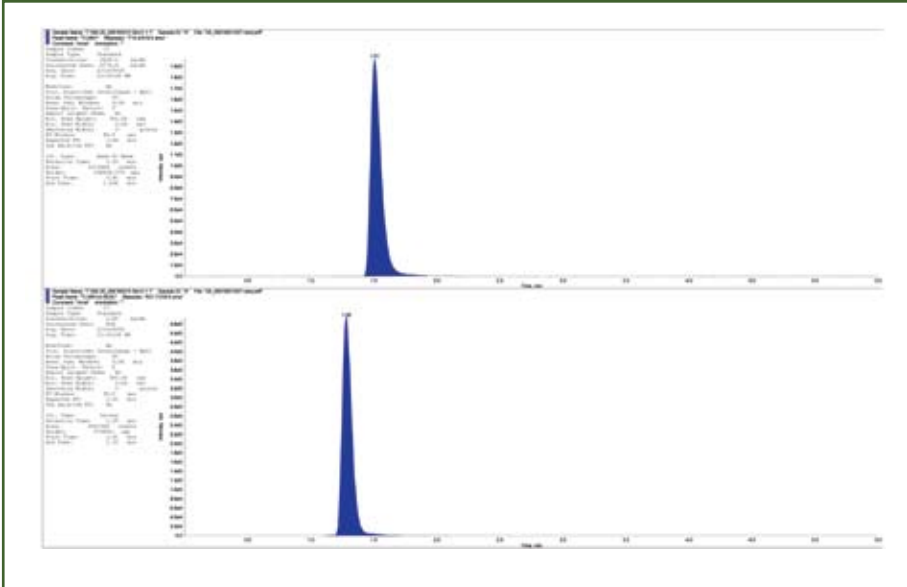


FIGURE 8. Representative Low Standard (10.0 ng/mL) for TL0901



## Results and Discussion continued

FIGURE 9. Representative High Standard (2,500 ng/mL) for TL0901



**TABLE 1:**  
Intra-Assay Accuracy and Precision for TL0901

Nominal Conc.	LLOQ QC 10.0 ng/mL	Low QC 30.0 ng/mL	Medium QC 250 ng/mL	High QC 2000 ng/mL	Dilution QC 6000 ng/mL
#1	10.2	31.7	256	2010	5910
#2	9.51	29.4	237	1940	6140
#3	10.4	27.6	249	1960	5830
#4	9.47	31.4	244	1770	5780
#5	9.77	26.4	223	1960	5520
#6	9.78	27.7	272	2020	5470
Mean	9.86	29	247	1940	5780
S.D.	0.373	2.17	16.7	90.5	250
%CV	3.8	7.5	6.8	4.7	4.3
%Theoretical	98.6	96.7	98.8	97	96.3
%Bias	-1.4	-3.3	-1.2	-3	-3.7
n	6	6	6	6	6



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## Conclusion

A robust, sensitive and specific method was developed to quantitatively determine the levels of an 18-mer phosphorothiate oligonucleotide in dog plasma. Similar assays have been successfully validated and used to analyze pre-clinical and clinical samples. All assessments for Incurred Sample Reanalysis (ISR) have passed demonstrating excellent reproducibility of the methods.

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## References

1. Guy A Tremblay and Philip R Oldfield, "Bioanalysis of siRNA and oligonucleotide therapeutics in biological fluids and tissues", *Bioanalysis* (2009), 1(3), 595-609.
2. Yanhui Zhang, Laixin Wang, Jian Chen, Yue Zhao, Min Meng and Patrick Bennett (Tandem Labs) as well as Donna Dobinson and Colin Green (Antisoma Research Limited, Welwyn Garden City, UK), "Quantitative determination of AS1411 oligonucleotide in human urine using UPLC-PDA", *the 11th Annual Symposium on Chemical & Pharmaceutical Structure Analysis, October 2008, Langhorne, PA.*
3. Jian Chen, Laixin Wang, Yanhui Zhang, Ming Meng, Juan Wang and Patrick Bennett (Tandem Labs) as well as Donna Dobinson and Colin Green (Antisoma Research Limited, Welwyn Garden City, UK), "Quantitative determination of AS1411 oligonucleotide in monkey plasma using UPLC-PDA", *the ISSX 15th Annual Meeting, October 2008, San Diego, CA.*
4. Laixin Wang, Weiwei Yuan, Yue Zhao, Jian Chen, Gregory vonArx, Min Meng and Patrick Bennett, "Quantitation of TL0901 Oligonucleotide in Human Plasma Using LC MS/MS", *AAPS Annual Meeting And Exposition, November 8 - 12, 2009, Los Angeles, CA.*