



# Comparison of Liquid/Liquid and Solid-Phase Extraction for the Quantitative Analysis of Phosphorothioate Oligonucleotides in Human Plasma Using Lc/Ms/Ms

## Authors

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## Introduction

Oligonucleotide therapeutics 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. Hybridization based immunoassays, HPLC-UV and CPG-UV have been predominately used for the quantitative analysis of oligonucleotides in support of TK/PK evaluations [1]. However, the immunoassays generally have narrow calibration ranges, poor specificity and low precision. HPLC-UV and CPG-UV methods typically have low sensitivity and long run times. Although the use of UPLC can reduce run times, the UV detection sensitivity is still not adequate for most studies [2, 3].

To overcome these shortcomings, we have developed and validated more than a dozen LC-MS/MS assays to analyze DNA oligonucleotides and siRNAs in biological matrices over the last three years. Both liquid-liquid extraction (LLE) and solid phase extraction (SPE) methods were evaluated for all tested oligonucleotides, the merits of each extraction being largely dependent on the structures of the oligonucleotides. Generally speaking, siRNA have better recovery on SPE extractions and phosphorothioate DNA oligonucleotides have better recoveries on LLE extractions. The linear ranges of the validated plasma assays are 10.0-2,500 ng/ml for single stranded DNA oligonucleotides and 25.0-5,000 ng/mL for double stranded siRNAs.

Here we present these methodologies by using a model 18-mer phosphorothioate DNA oligonucleotide (TL0901) with extraction from 200  $\mu$ L of human plasma.

## Methodology

### SAMPLE PREPARATION:

#### 1) SPE extraction

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 (1.0 M AmBicarb pH unadj)/water/MeCN/THF (v/v/v/v)] into a tapered 96-well plate which has been prefilled 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).

#### 2) LLE extraction

1. Thaw the samples in a wet ice-bath.
2. Aliquot 200  $\mu\text{L}$  of samples into the corresponding labeled 2.0 mL Eppendorf centrifuge tubes.
3. Add 50.0  $\mu\text{L}$  ice-cold working internal standard solution [10.0  $\mu\text{g}/\text{mL}$  of TL0901 (n-6) in water].
4. Add 200  $\mu\text{L}$  of pH=8.0 extraction buffer to all samples.
5. Add 200  $\mu\text{L}$  of the phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) solution to all samples.
6. Vortex-mix the samples thoroughly.
7. Centrifuge the samples in the microcentrifuge at 13,000 rpm for 15 minutes.
8. Transfer the clear supernatant to corresponding wells of 96-well plate and dried down.
9. Reconstitute the samples with 200  $\mu\text{L}$  mobile phase A.

## Methodology continued

### CHROMATOGRAPHIC CONDITIONS

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

Mobile Phase: A: HFIP and TEA buffered water

B: HFIP and TEA buffered MeOH

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

Injection volume: 5 – 20  $\mu$ L

Column temperature: 55°C

Flow rate: 0.300 mL/min

AS Temperature: RT

### 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 phosphorothioate oligonucleotide (sequence is 5'-ACTGTACG-ATTCGACCTA) that was randomly selected by Tandem Labs for the purpose of evaluating the analytical methods. A 12-mer analog phosphorothioate 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).
- For phosphorothioate DNA oligonucleotides, the extraction recoveries of the LLE extractions are ~80-87% from plasma samples. When SPE extraction is used, the recovery of TL0901 is comparable to the LLE, but the extraction recovery decreases significantly when the length of the phosphorothioate DNA oligonucleotide increases. For siRNA oligonucleotides, the SPE extractions usually provide recoveries of ~ 65-88%. Very poor recoveries (<20%) were obtained when the siRNA were extracted using the above LLE method.

### METHOD COMPARISON

- Two full accuracy and precision batches were extracted using either LLE or SPE method and analyzed under the same LC-MS/MS conditions. Each batch contains two calibration curves and 4 levels of quality controls (QC) at n=6 each. Both extraction methods obtained similar extraction recoveries for TL0901, but the LLE method had better precision (table 1 and 2). Divergent curves were also observed for the SPE extracted samples indicating the extracts were not as "clean" as the LLE extracts (Figure 6).

## Results and Discussion continued

FIGURE 1. Typical Q1 scan Mass Spectrum of TL0901.

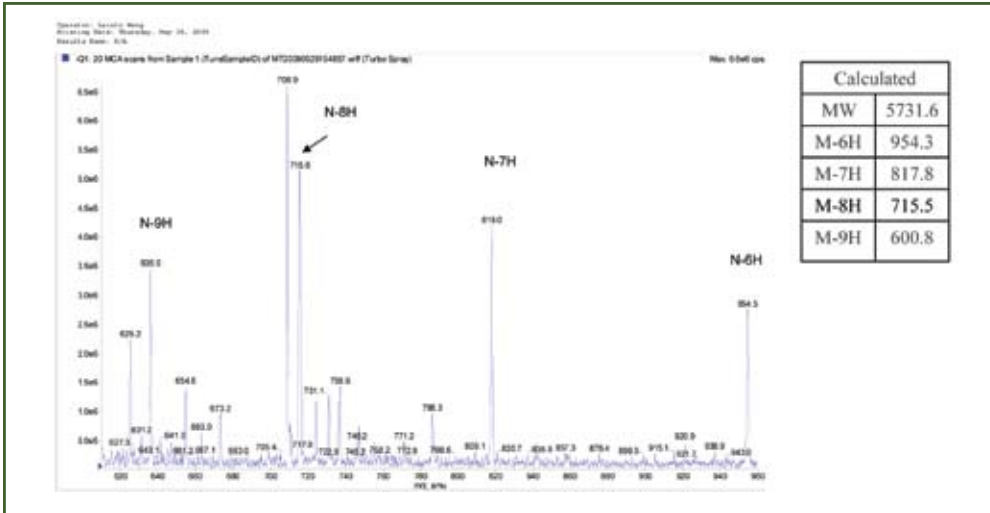
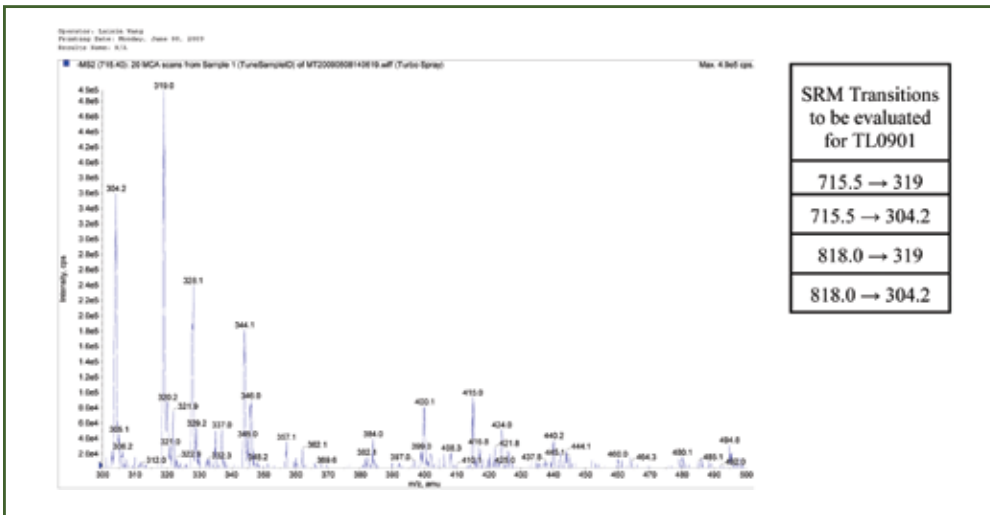


FIGURE 2. Typical Product Ion Scan Spectrum of TL0901



## Results and Discussion continued

FIGURE 3. Liquid Chromatographic Separation of TL0901 and Its n-4 and n-6 Metabolites

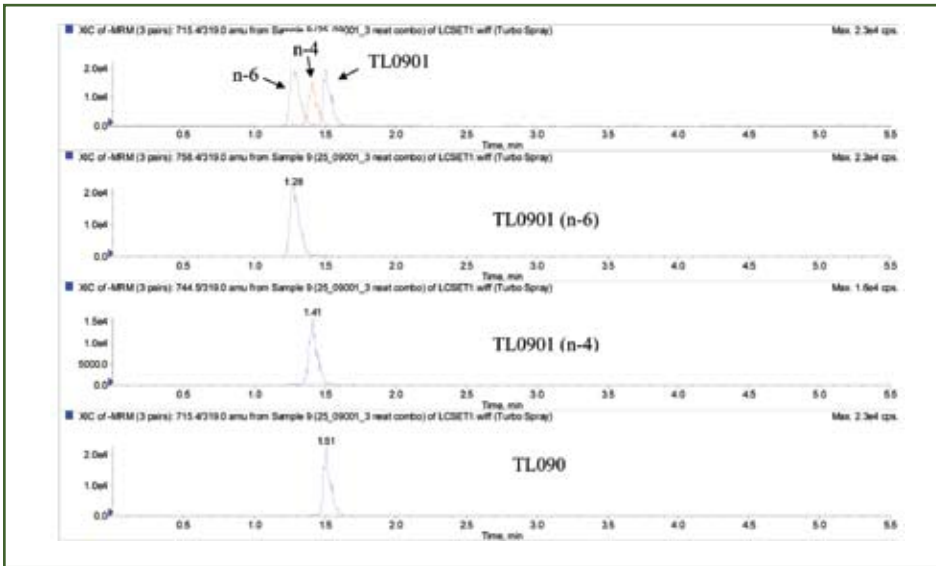
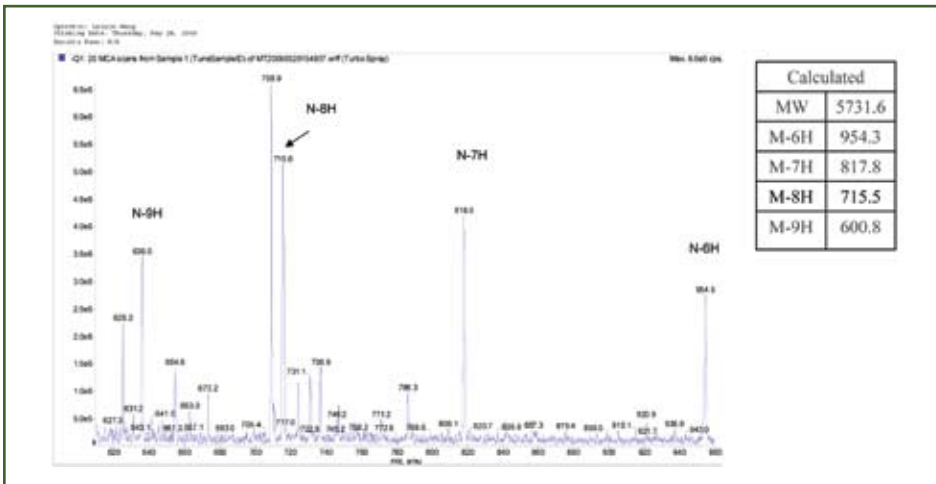


FIGURE 4. Typical Q1 scan Mass Spectrum of TL0901.



## Results and Discussion continued

FIGURE 5. The Calibration Curves (10.0-2,500 ng/mL) of LLE extracts

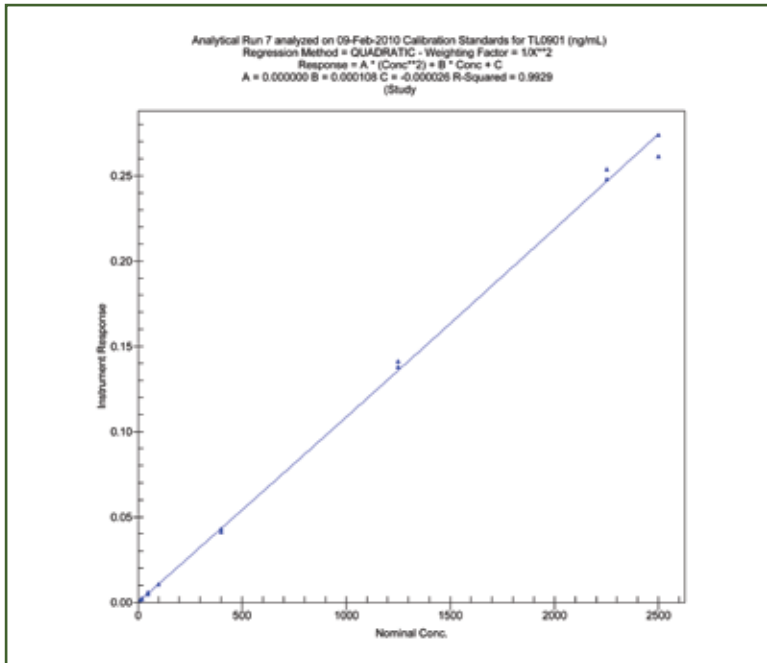
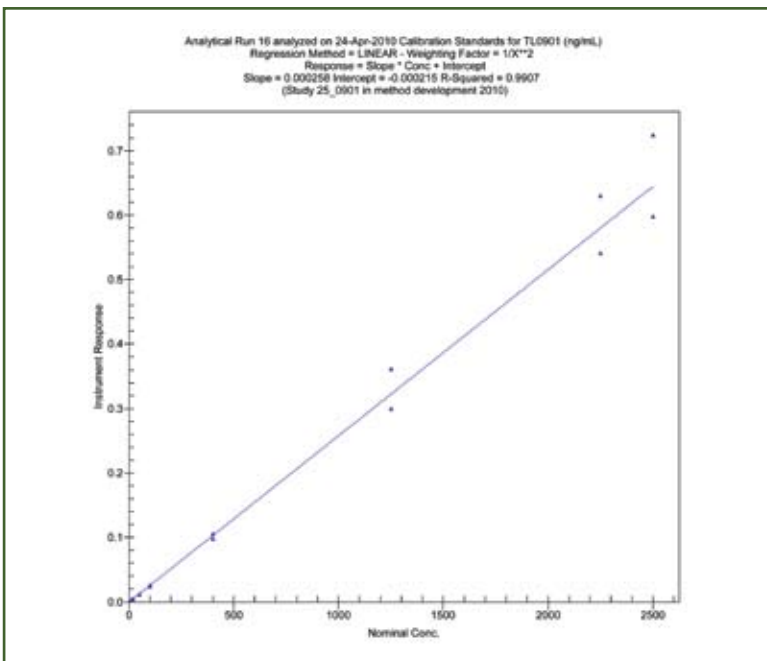


FIGURE 6. The Calibration Curves (10.0-2,500 ng/mL) of SPE extracts





## Results and Discussion continued

FIGURE 7. Representative Chromatogram of a Human Plasma Blank from LLE extracts

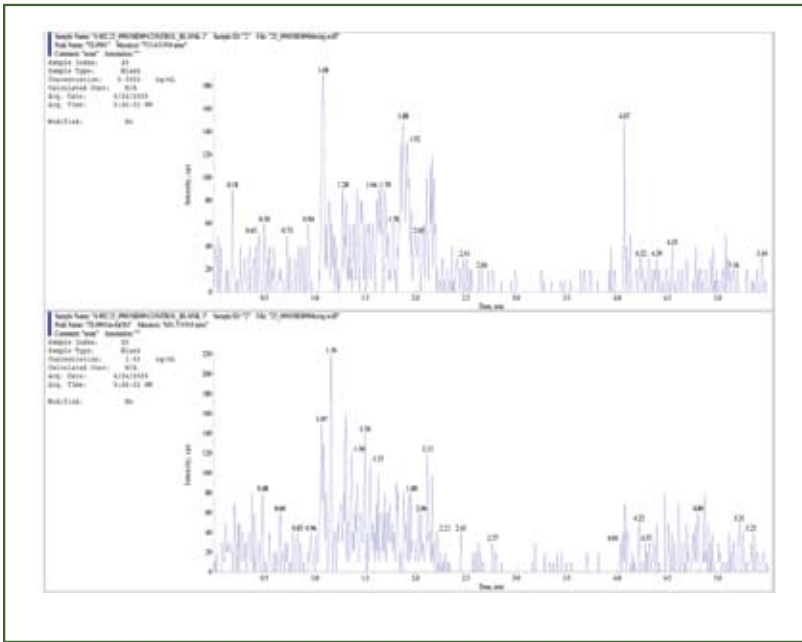
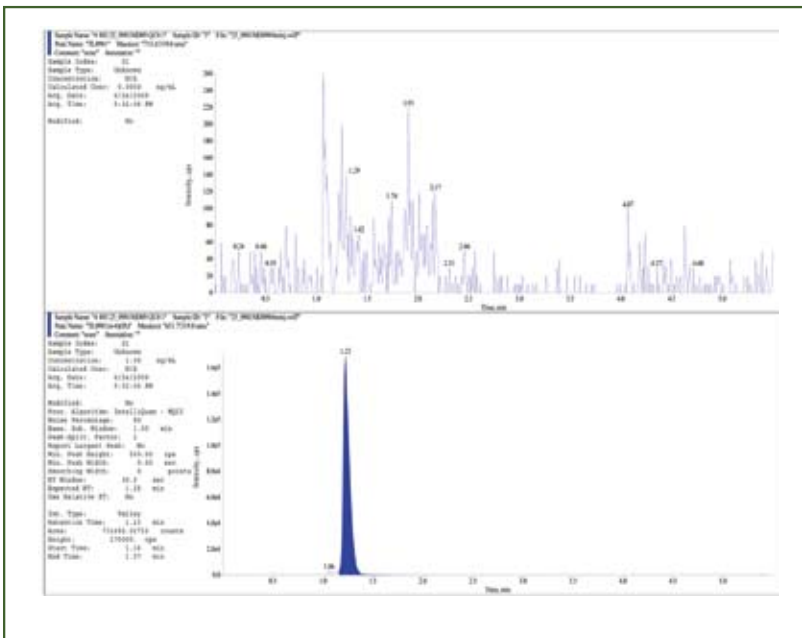


FIGURE 8. Representative Blank with Internal Standard (0-ng/mL QC) from LLE extracts



## Results and Discussion continued

FIGURE 9. Representative Low Standard (10.0 ng/mL) for TL0901 From LLE extracts

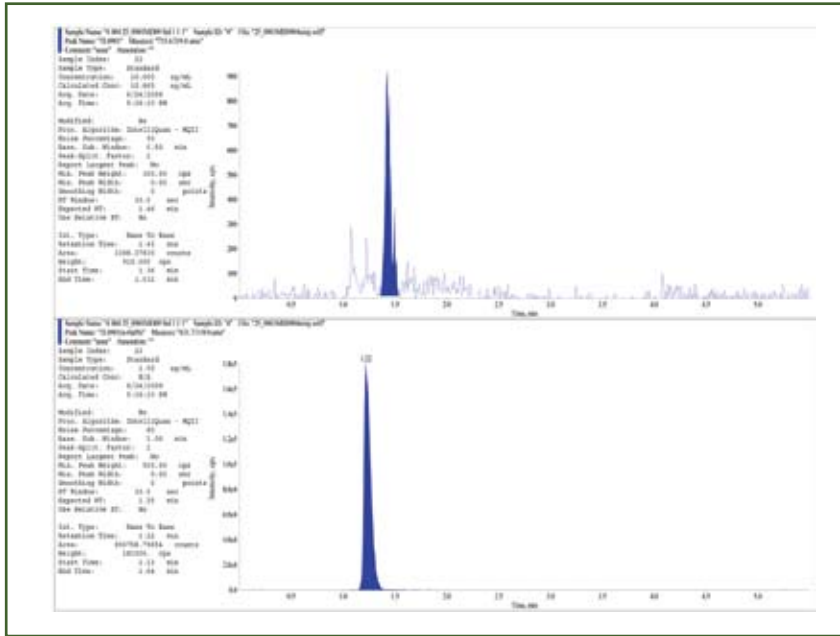
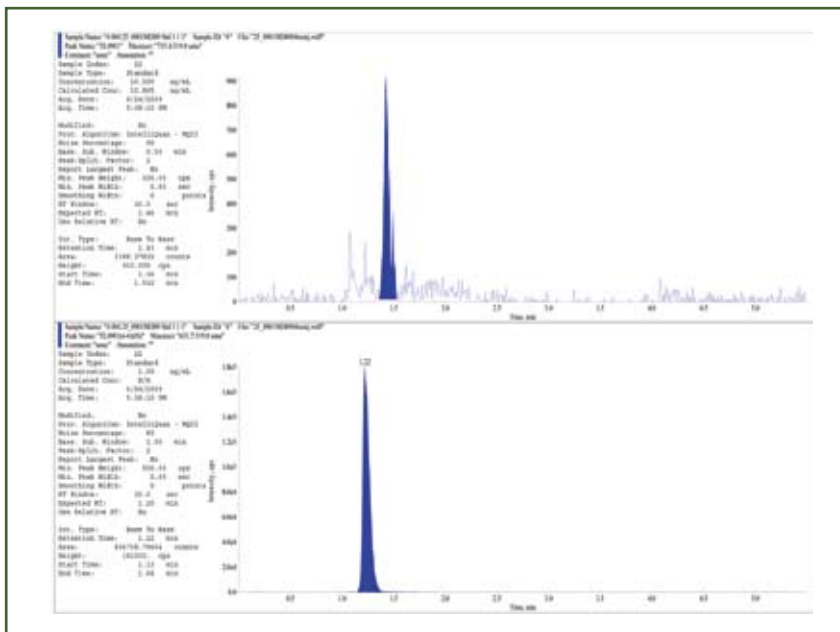


Figure 10. Representative High Standard (2,500 ng/mL) for TL0901 from LLE extracts



## Results and Discussion continued

**TABLE 1:**  
**Intra-Assay Accuracy and Precision for TL0901 from a LLE batch**

Nominal Conc.	LLOQ QC 10.0 ng/mL	Low QC 30.0 ng/mL	Medium QC 250 ng/mL	High QC 2000 ng/mL
#1	10.2	31.7	256	2010
#2	9.51	29.4	237	1940
#3	10.4	27.6	249	1960
#4	9.47	31.4	244	1770
#5	9.77	26.4	223	1960
#6	9.78	27.7	272	2020
Mean	9.86	29.0	247	1940
S.D.	0.373	2.17	16.7	90.5
%CV	3.80	7.50	6.80	4.70
%Theoretical	98.6	96.7	98.8	97.0
%Bias	-1.40	-3.30	-1.20	-3.00
n	6	6	6	6

**TABLE 2:**  
**Intra-Assay Accuracy and Precision for TL0901 from a SPE batch**

Nominal Conc.	LLOQ QC 10.0 ng/mL	Low QC 30.0 ng/mL	Medium QC 250 ng/mL	High QC 2000 ng/mL
#1	10.8	31.8	233	1930
#2	10.0	30.0	235	2020
#3	11.8	29.3	231	1950
#4	10.5	29.5	233	1710
#5	10.3	29.8	228	1760
#6	12.0	30.4	226	1840
Mean	10.9	30.1	231	1870
S.D.	0.820	0.903	3.41	119
%CV	7.50	3.00	1.50	6.40
%Theoretical	109	100.3	92.4	93.5
%Bias	9.00	0.300	-7.60	-6.50
n	6	6	6	6

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## Conclusion

Sensitive, specific and high throughput methods were developed to quantify the levels of an 18-mer phosphorothiate oligonucleotide in human plasma. Currently the LLE provides better slightly precision; however, the SPE extraction is easier to automate and therefore is capable of higher throughput. Similar assays have been successfully validated and used to analyze pre-clinical and clinical samples.

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## Reference

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, Min 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.