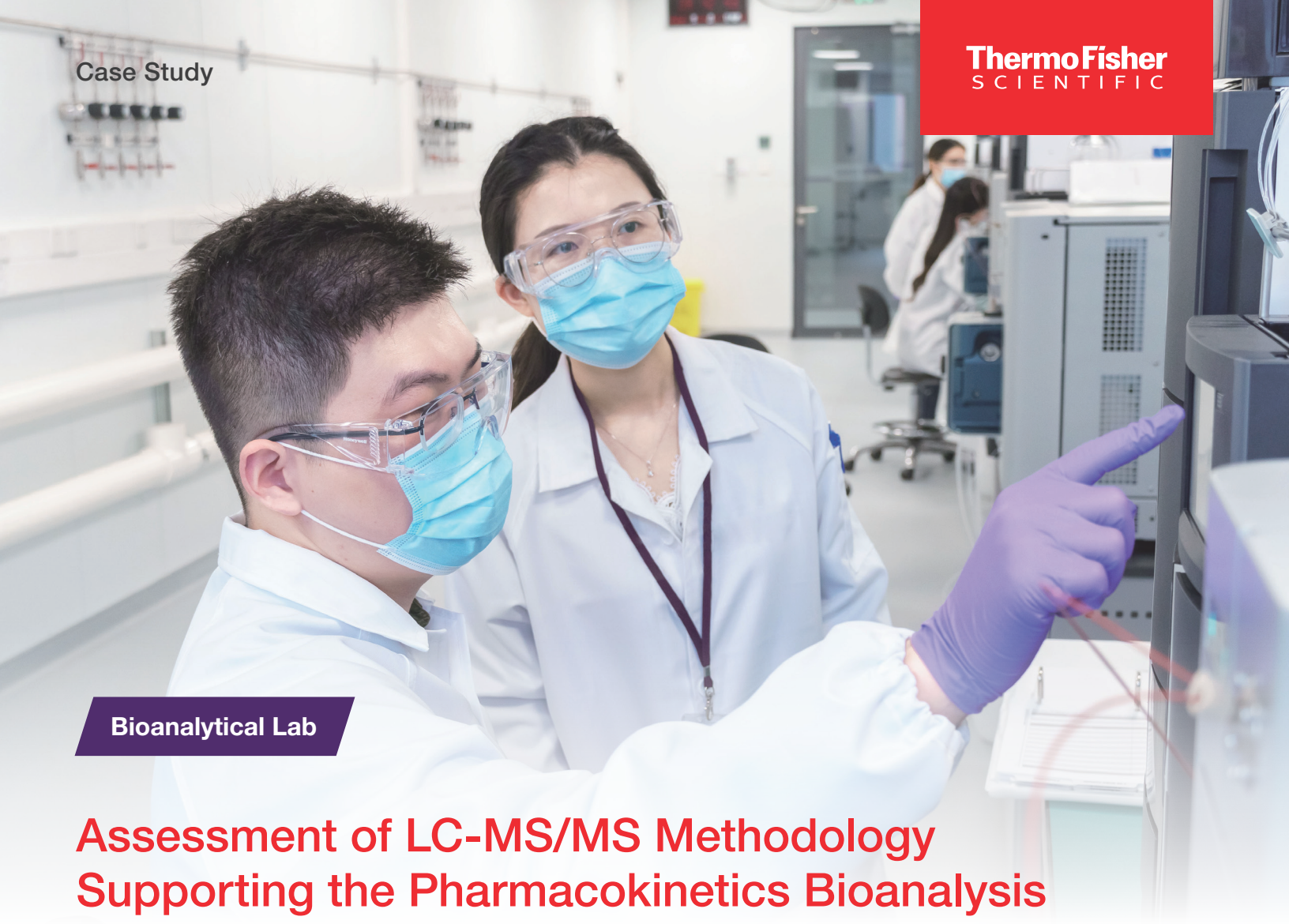
Case Study



Bioanalytical Lab

Assessment of LC-MS/MS Methodology Supporting the Pharmacokinetics Bioanalysis of Therapeutic siRNA

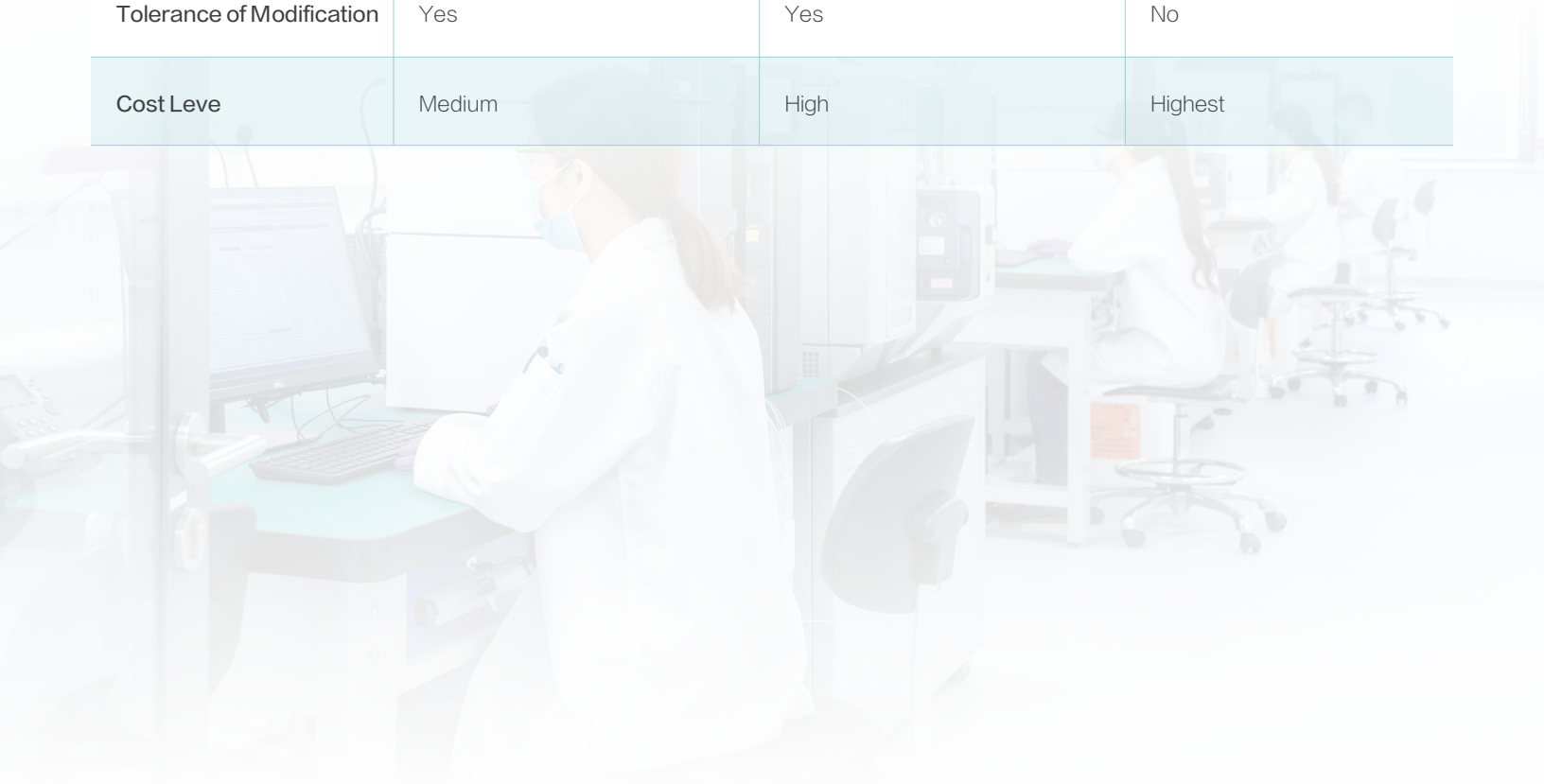
Considering the sensitivity, specificity, and quantitative analysis of metabolite analytical methods, the main analysis platform for pharmacokinetic bioanalysis of oligonucleotides is LC-MS (LC-MS/MS, LC-HRMS), supplemented by other analysis platforms. Oligonucleotides are short, single- or double-stranded DNA or RNA molecules (8–50 nucleotides in length), including antisense oligonucleotides (ASO), siRNA, miRNA, and aptamer RNAs. The PK bioanalysis of siRNA is challenging. siRNA is a double-stranded RNA with 22 to 25 base pairs (bp) that targets mRNA to trigger specific gene silencing and target degradation.

PPD™ Laboratory services bioanalytical lab has supported the bioanalysis of oligonucleotides in many pharmacokinetic clinical trials. In our bioanalytical team in Suzhou, China, we have developed and validated two robust bioanalytical methods for the analysis of anti-sense strand (AS) and its metabolites from siRNA in human plasma and urine by LC-MS/MS, the advantages of the methods are:

- **Highest specificity (molecular weight identification; production characterization; LC separation)**
- **High throughout (3-5 mins per injection)**
- **Wide dynamic range (500-1000x)**
- **Good sensitivity (5-10 ng/mL)**
- **No enzymes or special reagents needed**
- **Good reproducibility (±20% for LLOQ, ±15% for others)**

Comparison of Different Methodologies

Type	LC-MS	Hybridization ELISA/ECL	qRT-PCR
Sensitivity (LLOQ)	Medium (~5.00 ng/mL to ~10.00 ng/mL)	High (~0.100 ng/mL to ~1.00 ng/mL)	Highest (fg/mL to pg/mL)
Specificity	Highest (Less endogenous interference and less potential metabolites interference)	High (Endogenous interference)	Medium
Key Reagent	No need	Customized complementary probes	Customized complementary primers and probes
Metabolites Quantitation/ Identification	Yes	Yes	No
Sample Treatment	Need	Depending on methods	Need
Difficulty of R&D	Medium	High	最高
Tolerance of Modification	Yes	Yes	No
Cost Leve	Medium	High	Highest



Methods and Results

The siRNA molecule consists of a sense/antisense duplex. The sense strand is a 21-mer with GalNAc conjugation on the 3' end, and the antisense strand is a 23-mer. AS-Met is a 3', N-1 metabolite of the antisense strand. The siRNA duplex molecules are enriched using SPE, and then the duplexes undergo in-line chromatographic melting, which releases the sense and antisense strands via LC-MS/MS. The antisense strand is used for quantitation.

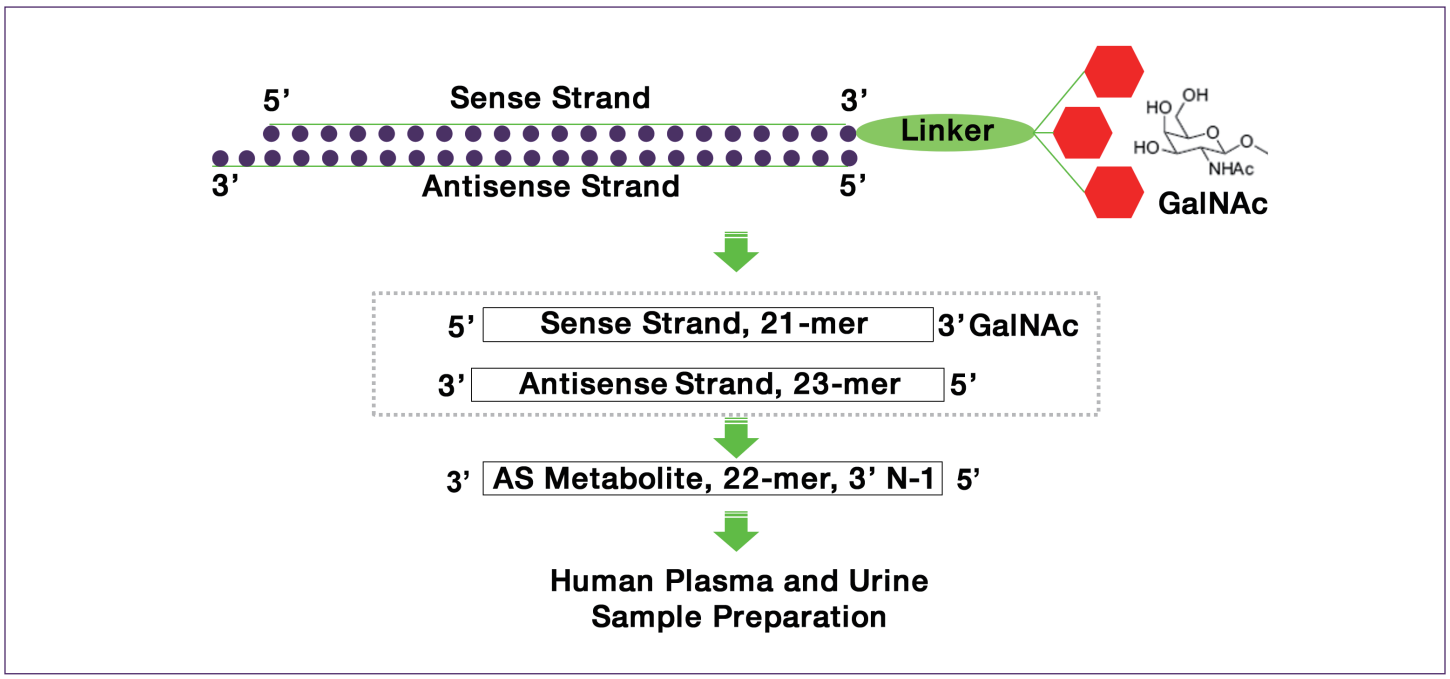


Fig1. Scheme of siRNA and Metabolite

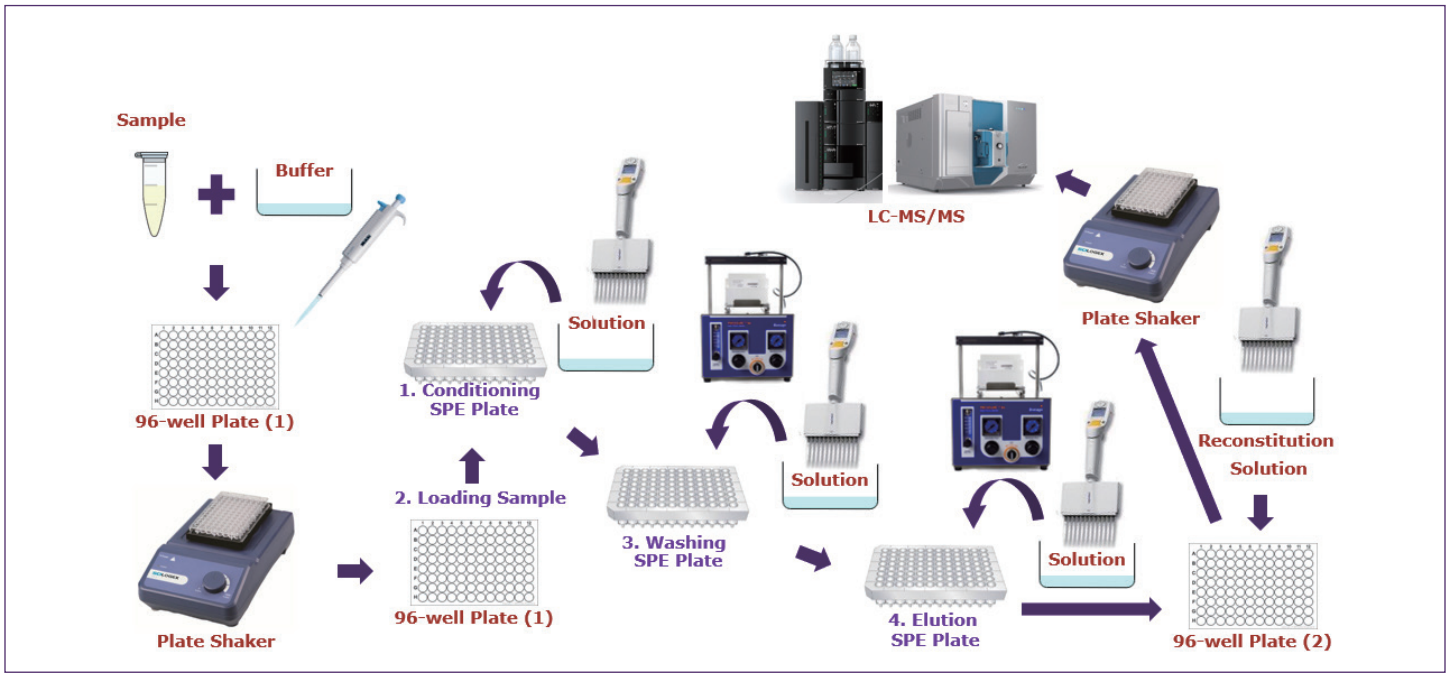


Fig2. Sample Preparation Procedure

Case 1:

Matrix: Human Plasma
Range: 2.00 - 1000 ng/mL
weighted linear regression curve (1/concentration2)
Sample Aliquot: 30.0 µL
Extraction: SPE

The anti-sense strand (AS) and metabolite extraction recovery in human plasma is close to 100% with Oligo SPE, and the AS and metabolite were stable in human whole blood.

Anti-sense strand (AS) SPE Extraction Recovery in Human Plasma

Duplicates	AS Area					
	PRE1	POST1	PRE2	POST2	PRE3	POST3
1	33440	29310	2367000	2448000	3435000	3601000
2	34640	33360	2522000	2427000	3627000	3488000
3	32300	31660	2491000	2420000	3484000	3613000
Mean	33460	31443	2460000	2431667	3515333	3567333
CV%	3.50	6.47	3.33	0.60	2.84	1.93
RE%		**106.41**		**101.17**		**98.54**

Anti-sense strand (AS) Metabolite SPE Extraction Recovery in Human Plasma

Duplicates	AS-Met Area					
	PRE1	POST1	PRE2	POST2	PRE3	POST3
1	30830	30490	2147000	2213000	3119000	3016000
2	30560	31160	2188000	2175000	2917000	3029000
3	30070	29240	2128000	2189000	2986000	2895000
Mean	30487	30297	2154333	2192333	3007333	2980000
CV%	1.26	3.22	1.42	0.88	3.41	2.48
RE%		**100.63**		**98.27**		**100.92**

Anti-sense strand (AS) Stability in Human Whole Blood

Duplicates	AS/IS Area Ratio					
	WB-QC1 -RT-CRT 0h	WB-QC1 -RT-CRT 2h	WB QC1 -RT-CC 0h	WB QC1 -RT-CC 2h	WB QC1 -4C-CRT 2h	WB QC1 -4C-CC 2h
	6 ng/ml in whole blood					
1	0.0358	0.0340	0.0314	0.0331	0.0300	0.02983
2	0.0344	0.0336	0.0336	0.0329	0.0291	0.02923
3	0.0310	0.0308	0.0331	0.0315	0.0286	0.03015
Mean	0.0337	0.0328	0.0327	0.0325	0.0292	0.0297
CV%	7.34	5.28	3.56	2.61	2.45	1.57
RE%		**97.40**		**99.38**	**86.69**	**90.93**

Duplicates	AS/IS Area Ratio					
	WB-QC3 -RT-CRT 0h	WB-QC3 -RT-CRT 2h	WB QC3 -RT-CC 0h	WB QC3 -RT-CC 2h	WB QC3 -4C-CRT 2h	WB QC3 -4C-CC 2h
	750 ng/ml in whole blood					
1	3.3690	3.4430	3.4400	3.2410	3.4320	3.328
2	3.3680	3.4310	3.5690	3.5280	3.5040	3.441
3	3.3150	3.4940	3.4710	3.3570	3.5700	3.423
Mean	3.3507	3.4560	3.4933	3.3753	3.5020	3.3973
CV%	0.92	0.97	1.93	4.28	1.97	1.79
RE%		**103.14**		**96.62**	**104.52**	**97.25**

Anti-sense strand (AS) Metabolite Stability in Human Whole Blood

Duplicates	AS/IS Area Ratio					
	WB-QC1 -RT-CRT 0h	WB-QC1 -RT-CRT 2h	WB QC1 -RT-CC 0h	WB QC1 -RT-CC 2h	WB QC1 -4C-CRT 2h	WB QC1 -4C-CC 2h
	6 ng/ml in whole blood					
1	0.0539	0.0492	0.0528	0.0502	0.0497	0.04803
2	0.0531	0.0546	0.0523	0.0511	0.0510	0.04841
3	0.0524	0.0525	0.0493	0.0494	0.0490	0.04861
Mean	0.0531	0.0521	0.0515	0.0502	0.0499	0.0484
CV%	1.40	5.22	3.67	1.71	2.05	0.61
RE%		**98.07**		**97.60**	**93.95**	**93.97**

Duplicates	AS/IS Area Ratio					
	WB-QC3 -RT-CRT 0h	WB-QC3 -RT-CRT 2h	WB QC3 -RT-CC 0h	WB QC3 -RT-CC 2h	WB QC3 -4C-CRT 2h	WB QC3 -4C-CC 2h
	750 ng/ml in whole blood					
1	5.5870	5.6530	5.7250	5.6750	5.6940	5.642
2	5.6800	5.6460	5.8830	5.7460	5.8790	5.798
3	5.7030	5.7850	5.8390	5.7950	5.9890	5.842
Mean	5.6567	5.6947	5.8157	5.7387	5.8540	5.7607
CV%	1.09	1.38	1.40	1.05	2.55	1.82
RE%		**100.67**		**98.68**	**103.49**	**99.05**

Case 2:

Matrix: Human Urine
Range: 50.0 - 20000 ng/mL
weighted linear regression curve (1/concentration2)
Sample volume: 30.0 µL
Extraction: SPE

The anti-sense strand (AS) and metabolite extraction recovery in human urine is close to 100% with Oligo SPE.

Anti-sense strand (AS) SPE Extraction Recovery in Human Urine

Duplicates	AS Area					
	PRE1	POST1	PRE2	POST2	PRE3	POST3
1	98480	106500	1508000	1625496	2138000	2239000
2	94090	95660	1501000	1644186	2157000	2230000
3	95820	100800	1469000	1587938	2116000	2204000
Mean	96130	100987	1492667	1587938	2137000	2224333
CV%	2.30	5.37	1.39	1.77	0.96	0.82
RE%		**95.19**		**92.19**		**96.07**

Anti-sense strand (AS) Metabolite SPE Extraction Recovery in Human Urine

Duplicates	AS-Met Area					
	PRE1	POST1	PRE2	POST2	PRE3	POST3
1	115400	129600	1783000	1958000	2564000	2655000
2	110300	122100	1786000	1918840	2571000	2708000
3	117400	124200	1800000	1883240	2553000	2617000
Mean	114367	125300	1789667	1920027	2562667	2660000
CV%	3.20	3.09	0.51	1.95	0.35	1.72
RE%		**91.27**		**93.21**		**96.34**

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