APPLICATIONS

A Fast and Effective Quantitation Method for Uracil, 5,6-Dihydrouracil, and 5-Fluorouracil from Human Serum by LC-MS/MS

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Introduction

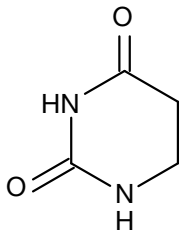
Uracil, 5-Fluorouracil (5FU), and 5,6-Dihydrouracil (UH_2) offer unique markers for enzymatic activity. In order to determine the 5FU catabolic rate, uracil and its homologues are tested in correlation with each other to ultimately determine if sufficient metabolization is occurring. The primary goal of this study was to develop a sample preparation and LC-MS/MS method for quantitative analysis of uracil and its two homologues from human serum. This application focuses on developing a sample preparation method and LC-MS/MS analysis using a Strata®-X PRO solid phase extraction (SPE) and a Kinetex® PS C18 HPLC column respectively. The Kinetex PS C18 column is a positively charged, surface modified C18 phase that caters its unique selectivity to separating the very polar uracil and its homologues in this analysis, while the novel Strata-X PRO greatly reduces the phospholipids in the sample and provides cleaner extracts.

Materials and Methods

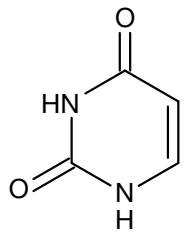
Reagents and Chemicals

Analytical reference standards and internal standards were purchased from Cerilliant® Corporation Corporation (Round Rock, TX, USA). Doubly stripped MS grade human serum and pooled human plasma K_2EDTA were obtained from Golden West Diagnostics and BioreclamationIVT® (Westbury, NY). All other reagents and chemicals were purchased from the Sigma-Aldrich® Company (St. Louis, MO). Ultrapure D.I. water was obtained from Sartorius arium® comfort I , courtesy of Sartorious Corporation (Bohemia, NY).

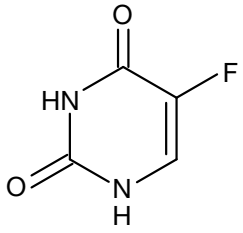
5,6-Dihydrouracil
Molar mass: 114.104 g/mol
Acidic pK_a: 11.73
LogP: -1.21



Uracil
Molar mass: 112.088 g/mol
Acidic pK_a: 8.8
LogP: -0.5



5-Fluorouracil
Molar mass: 130.078 g/mol
Acidic pK_a: 7.18
LogP: -0.66



SPE Procedure

Sample Pre-treatment: Add 100 µL Human serum (doubly stripped) to a tube. Add 900 µL of chilled (~0°C) Acetonitrile to the serum while vortexing. Centrifuge samples at 6000 rpm for 5 minutes.

96-Well Plate:	Strata-X PRO, 30 mg/well
Part No.:	8E-S536-TGA
Condition:	1 mL Acetonitrile
Load:	Pass the supernatant (From Pre-treatment) and apply vacuum to collect eluted extract
Dry Down:	Under Nitrogen and heat at ~45 °C
Reconstitute:	With 100 µL initial mobile phase Note: For phospholipid analysis (1) Human plasma K_2EDTA was utilized (2) Direct injection (bypass dry down and reconstitute) of the eluted sample was made.

Quantitative Analysis for Uracil Homologues

Column:	Kinetex 2.6 µm PS C18
Dimensions:	150 x 3.0 mm
Part No.:	00F-4780-Y0
Mobile Phase:	A: 0.1% Formic acid in Water B: Methanol
Gradient:	**Time (min)** **% B** 0 7 12 7
Flow Rate:	0.2 mL/min
Injection Volume:	10 µL
Column Temperature:	25 °C
Instrument:	Agilent® 1260
Detection:	MS/MS (SCIEX® Triple Quad™ 4500, ESI Dual Polarity) (Positive for U and UH_2, Negative for 5FU)

Qualitative Analysis for Phospholipids

Column:	Kinetex 2.6 µm C18
Dimensions:	50 x 2.0 mm
Part No.:	00B-4462-AN
Mobile Phase:	A: 0.1% Formic acid in Water B: 0.1% Formic acid in Methanol
Gradient:	**Time (min)** **% B** 0 40 0.5 95 11.5 95 11.51 40 13.5 40
Flow Rate:	0.4 mL/min
Injection Volume:	2 µL
Column Temperature:	40 °C
Instrument:	Agilent 1260
Detection:	MS/MS (SCIEX® Triple Quad™ 4500, ESI+)

Table 1.
Retention Time (RT), MRM Transition, and % Recovery for Analytes

Analyte	RT	Q1	Q3	Spiked conc. (ng/mL)	% Recovery	% CV
UH_2	4.31	114.9	55.04	100	90	5.4
U	4.65	112.9	69.8	100	84	3.2
5FU	7.07	128.8	41.9	100	89	3.2
			86.1			
			58.9			
Uracil 1,3-$15N_2$ (+Ve IS)	4.65	114.8	96.9	200	N/A	N/A
5 Cl Uracil (-Ve IS)	10.82	145.1	42.1	200	N/A	N/A

Figure 1.
Representative Chromatogram of Extracted Human Serum Analyzed by a Kinetex® 2.6 µm PS C18 LC Column (Under ESI Positive Polarity)

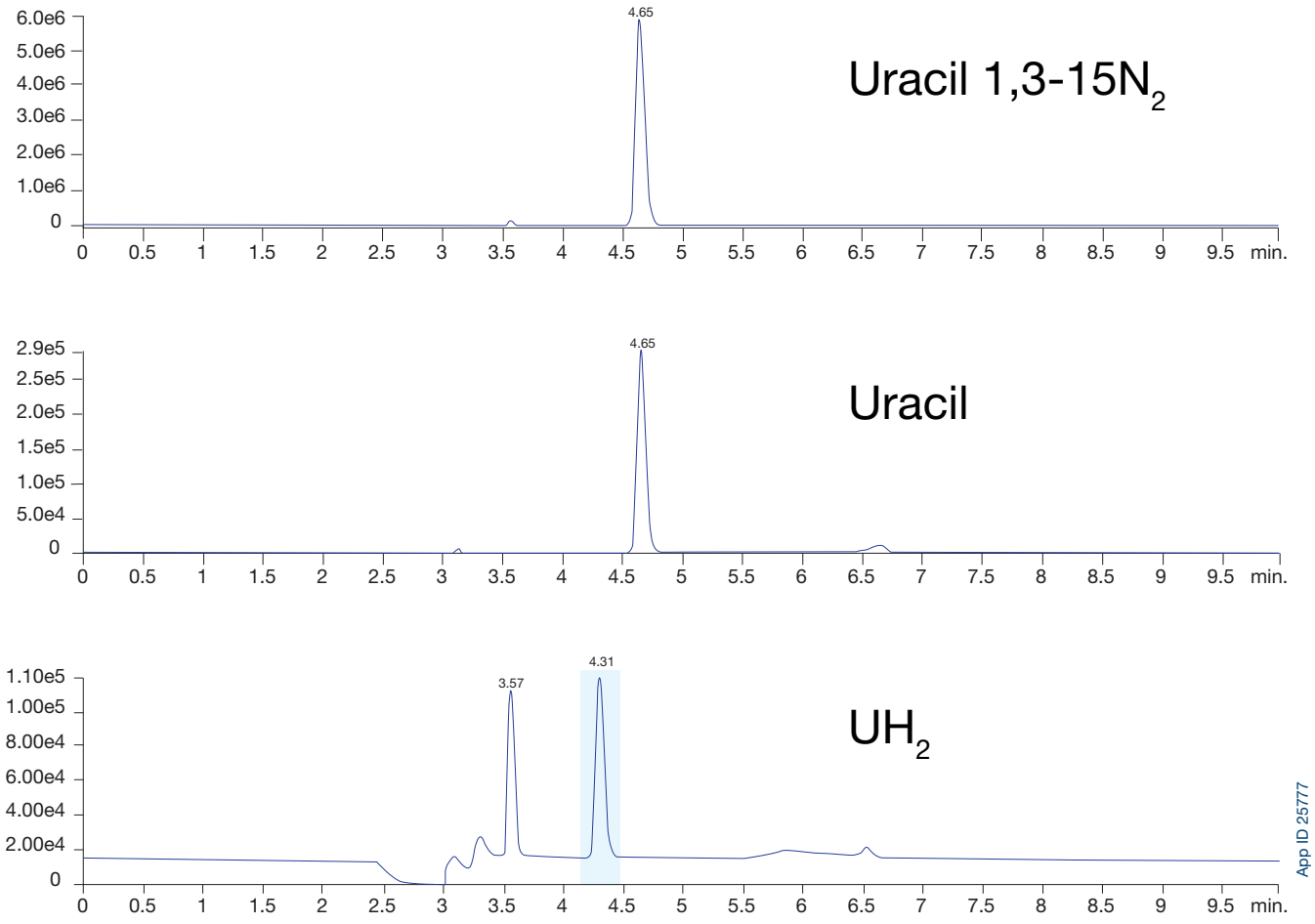


Figure 2.
Representative Chromatogram of Extracted Human Serum Analyzed by a Kinetex® 2.6µm PS C18 LC Column (Under ESI Negative Polarity)

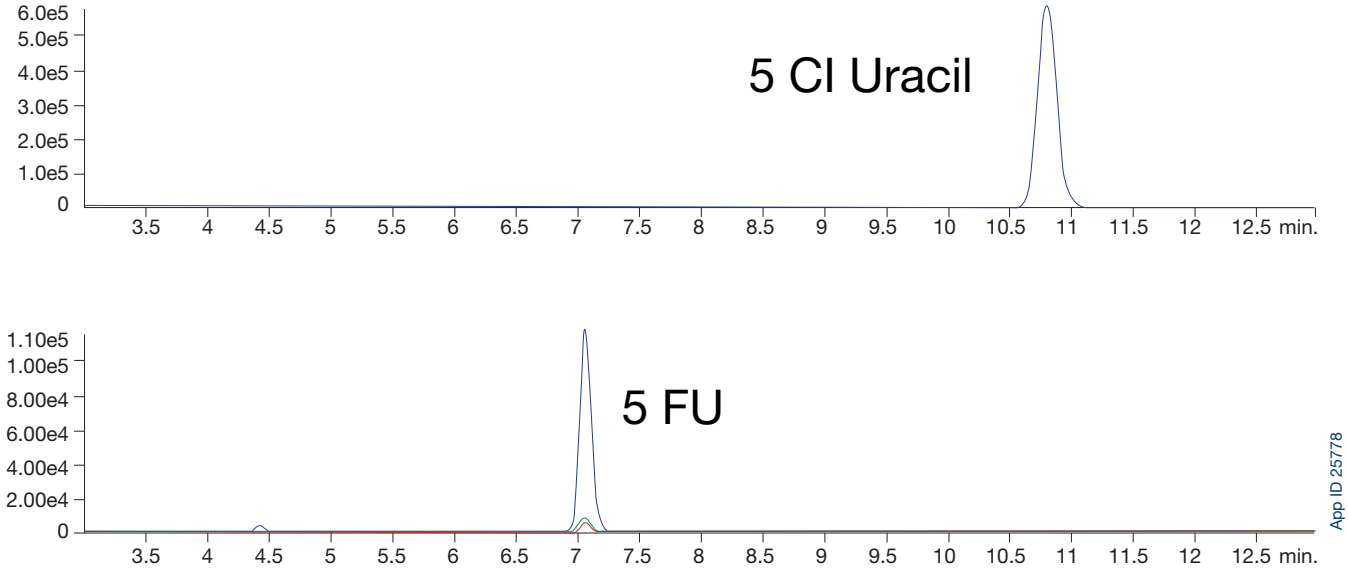


Figure 3.
Representative Chromatogram for Qualitative Evaluation of Phospholipid in Extracted Human Plasma Samples With Two Different Clean-up Techniques Employed (A) Protein Precipitation (B) Strata®-X PRO

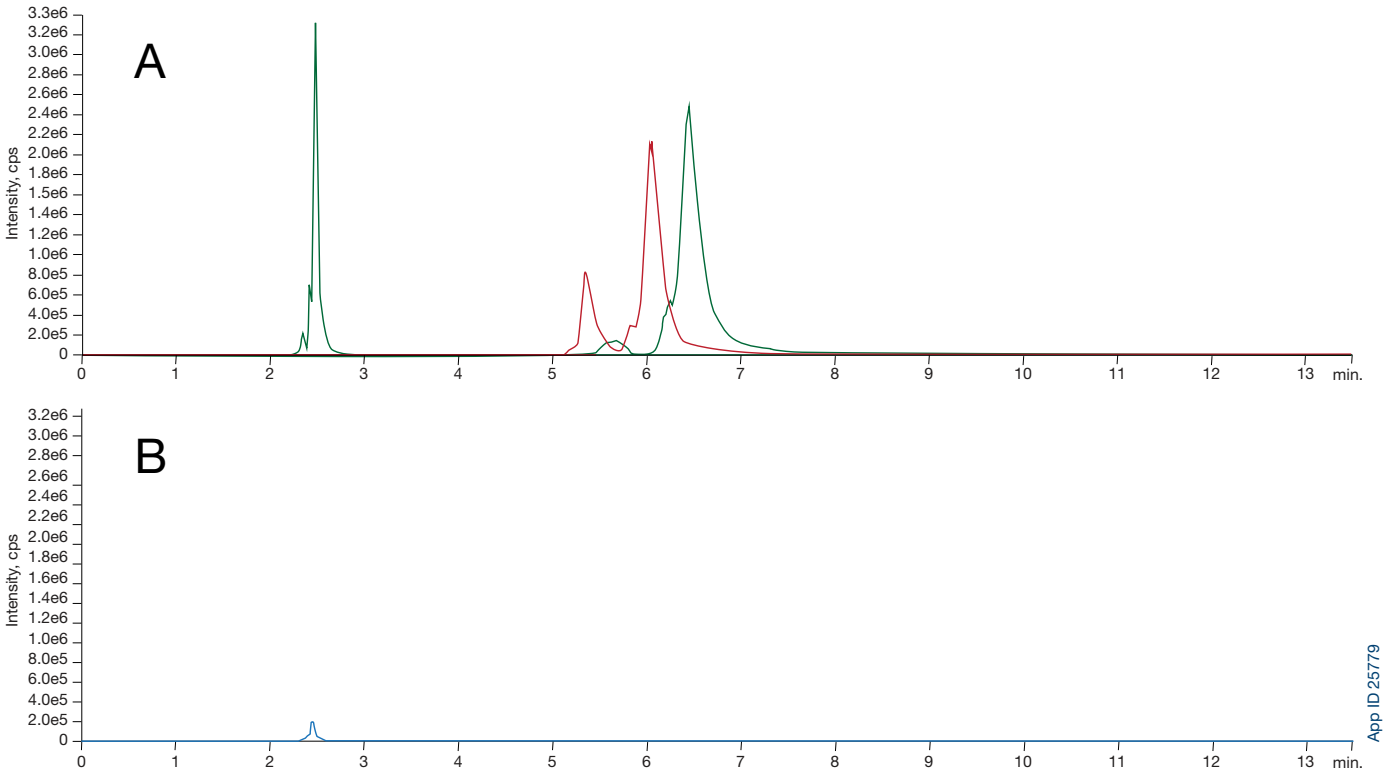


Figure 4.
Linearity Curve for Uracil Extracted Human Serum Over 200-fold (5 to 1000 ng/mL) Dynamic Concentration Range

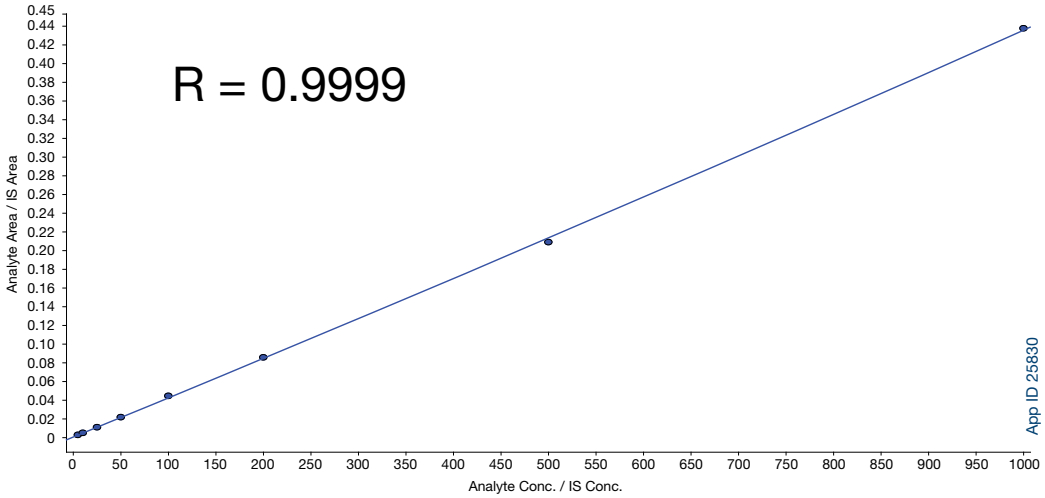


Figure 5.
Linearity Curve for UH_2 Extracted Human Serum Over 200-fold (5 to 1000 ng/mL) Dynamic Concentration Range

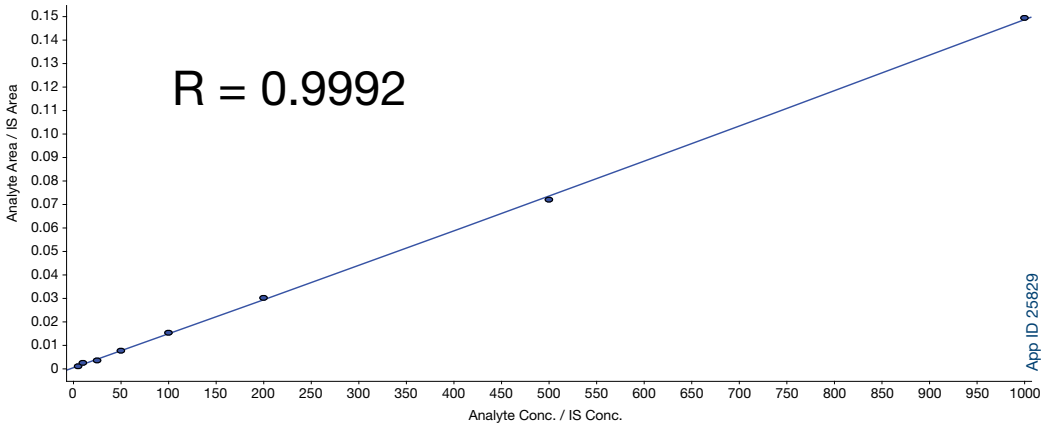


Figure 6.
Linearity Curve for 5FU Extracted Human Serum Over 200-fold (5 to 1000 ng/mL) Dynamic Concentration Range

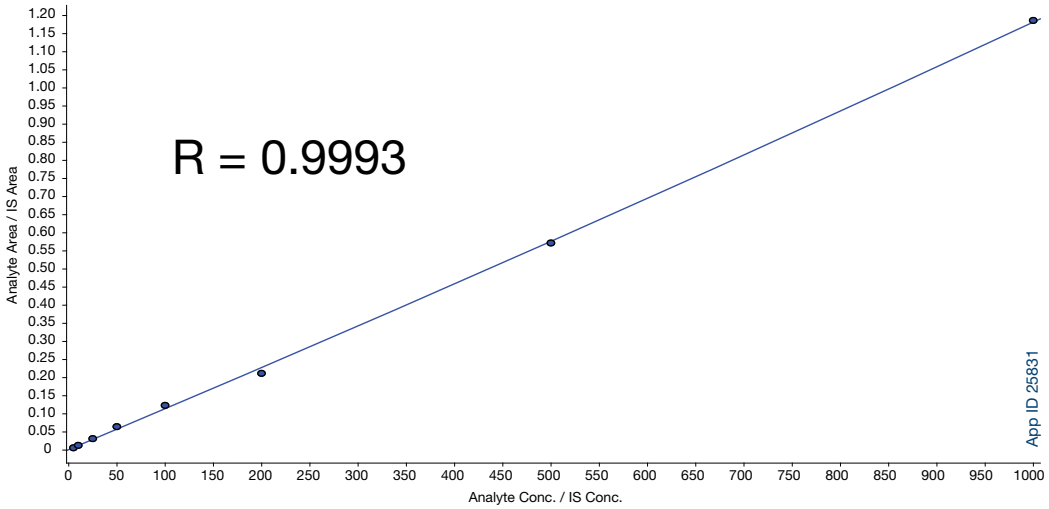


Table 2.
Precision and Accuracy Data for QC Samples of Uracil

Expected Conc. (ng/mL)	Sample	Replicates (N = 4)	%CV	Accuracy
30	QC1-1	4	4.6	97.4
150	QC1-2	4	5.3	101.6
750	QC1-3	4	6.9	102

Table 3.
Precision and Accuracy Data for QC Samples of UH_2

Expected Conc. (ng/mL)	Sample	Replicates (N = 4)	%CV	Accuracy
30	QC1-1	4	11.8	89.3
150	QC1-2	4	8.1	96.2
750	QC1-3	4	5.3	94.8

Table 4.
Precision and Accuracy Data for QC Samples of 5FU

Expected Conc. (ng/mL)	Sample	Replicates (N = 4)	%CV	Accuracy
30	QC1-1	4	2.4	106.7
150	QC1-2	4	10.1	105.0
750	QC1-3	4	7.8	108.9

Discussion

The unique chemistry offered by the positively charged Kinetex® PS C18 LC column retains the uracil and its homologues (UH_2 and 5FU) adequately to provide great resolution between these 3 compounds (**Figure 1 and 2**). The column selectivity allows the mass spec to utilize a polarity switch from positive to negative and simultaneously analyze all compounds in one run. The qualitative analysis demonstrates the superiority in cleanliness while sample obtained from protein precipitation undergoes extraction, utilizing Strata®-X PRO SPE (**Figure 3**). The selectivity of Strata-X PRO chemistry promotes elimination of majority of the phospholipids present in plasma sample, resulting in cleaner extract with minimum background noise. The absolute recoveries of the above three analytes in extracted samples yield more than 80% (**Table 1**). The QC samples for replicate extraction at 3 different levels showed precision and accuracy data between 2.4-11.8% and 89.3-108.9% respectively, that are within acceptable industry standard (**Table 2, 3, & 4**). The dynamic range of this method was tested with eight calibrators from 5 to 1000 ng/mL concentration range with an acceptable lin-earity ($r \geq 0.9995$) value (**Figure 4-6**).

Conclusion

The prescribed sample preparation method utilizing a protein precipitation followed by a 2 step Strata-X PRO extraction results in a simple and rapid quantitation of uracil and its analogues from human serum. The unique selectivity of the Kinetex PS C18 column demonstrated good chromatographic performance and overcame the challenges of analysis of these extremely polar compounds that the reversed phase columns encounter.

References

1. Barbara Buchel, Peter Rhyn, Stefan Schurch, *Biomed. Chromatogr.* **2013**; 27 : 7-16.
2. Ruta Svobaite, Isabella Solassol, Frederic Pinguet, *Clinical Chemistry* 54:9,1463-1472 (**2008**).
3. Jenny P. Dai, Amra Tabakovic, Welley Loc, *Current Trends in Mass Spectrometry* **October 2013**.

Kinetex® Core-Shell LC Column Ordering Information

2.6 µm Minibore Columns (mm)					SecurityGuard™ ULTRA Cartridges‡
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
PS C18	00A-4780-AN	00B-4780-AN	00D-4780-AN	00F-4780-AN	AJ0-8951 for 2.1 mm ID

2.6 µm MidBore™ Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 3.0	100 x 3.0	150 x 3.0	3/pk
PS C18	00B-4780-Y0	00D-4780-Y0	00F-4780-Y0	AJ0-8950 for 3.0 mm ID

2.6 µm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
PS C18	00B-4780-E0	00D-4780-E0	00F-4780-E0	00G-4780-E0	AJ0-8949 for 4.6 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000.

Ordering Information Strata®-X PRO SPE

Format	Sorbent Mass	Part Number	Unit
Tube			
	10 mg	8B-S536-AAK	1 mL (100/box)
	30 mg	8B-S536-TAK	1 mL (100/box)
	30 mg	8B-S536-TBJ	3 mL (50/box)
	60 mg	8B-S536-UBJ	3 mL (50/box)
	200 mg	8B-S536-FBJ	3 mL (50/box)
	100 mg	8B-S536-ECH	6 mL (30/box)
	200 mg	8B-S536-FCH	6 mL (30/box)
	500 mg	8B-S536-HCH	6 mL (30/box)
96-Well Plate			
	10 mg/well	8E-S536-AGA	ea
	30 mg/well	8E-S536-TGA	ea
	60 mg/well	8E-S536-UGA	ea
96-Well Microelution Plate			
	2 mg/well	8M-S536-4GA	ea

Presston™ 1000 Positive Pressure Manifold

Part No.	Description
AH1-7033	Presston 1000 Positive Pressure Manifold, 96-Well Plate

Phenomenex warrants the Presston 1000 will be free of defects in materials and workmanship under normal installation, use, and maintenance for a period of 12 months following delivery. **Please visit www.phenomenex.com/Presstonwarranty** for complete warranty information.

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