Maximising Analyte Recovery using the Phenomenex Phree™ Phospholipid Removal Plate with an Established LC/MS/MS Method using Kinetex® Core-Shell C18 HPLC/UHPLC Columns to Quantify 25-OH Vitamin D_2 and D_3

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This technical note presents preliminary data showing that simultaneously removing proteins and phospholipids from plasma samples with a Phenomenex Phree phospholipid removal plate showed a superior sample clean-up compared to a standard protein precipitation method. Quantification of 25-OH vitamin D and detection of LLOQ calibrators (5 nmol/L) was easier compared to samples where protein precipitation alone had been performed. This work was performed by the Royal Liverpool & Broadgreen University Hospital and was presented at the British Mass Spectrometry Society (BMSS) 2014 Annual Meeting.

Introduction

Abnormal concentrations of 25-OH vitamin D_3 in plasma are being linked to a number of serious medical conditions including musculoskeletal diseases, cancer, and diabetes. This has caused a huge year-on-year increase in the number of 25-OH vitamin D_3 analyses being conducted in clinical laboratories[1]. Traditionally immunoassay and HPLC/UV were the main techniques utilised, more recently laboratories have been increasingly employing the use of LC/MS/MS. Recent studies have shown that data from LC/MS/MS is more accurate and shows a greater level of specificity than by immunoassay[2]. Given the levels of sensitivity required to accurately quantify 25-OH vitamin D_3, LC/MS/MS is the only practical alternative.

A degree of sample preparation is required for the LC/MS/MS analysis of 25-OH vitamin D_3, otherwise there is a risk of blockage and contamination of HPLC columns and LC/MS/MS equipment. At minimum it is necessary to perform protein precipitation or a combination of protein precipitation and Solid Phase Extraction (SPE). Protein precipitation alone is less expensive and quicker; however, samples are not as clean as when using SPE. This makes quantitation at the LLOQ (5 nmol/L) or for severely deficient samples (< 15 nmol/L) a real challenge. Phospholipids are a major contributor to sample contamination, and it has been reported that they can suppress analyte signals and contaminate columns and systems[4].

Please note that the data shown in this technical note is for 25-OH vitamin D_3 however it should be noted 25-OH vitamin D_2 also behaved in the same way.

Method

Existing protein precipitation method:

Serum was precipitated using Zinc sulfate ($ZnSO_4$) and Acetonitrile (ACN) containing trideuterated 25-OH vitamin D_2 and D_3 internal standards. Analysed by reversed phase chromatography using a Phenomenex Kinetex core-shell 2.6 µm C18 100 Å 50 x 3 mm HPLC/UHPLC column on an ACQUITY® UPLC® separation module coupled to a Xevo™ TQS mass spectrometer (Waters Corp.). Details of the protein precipitation are as follows:

1. Thoroughly mix all samples before use.
2. Place 100 µL of calibration standards/QC/samples into each well.
3. Add 100 µL of zinc sulphate into each well.
4. Add 200 µL of working internal standard into all wells. Heat seal the plate with a plastic sheet, then place the plate on the plate shaker for 1 hr at 900 rpm.
5. Centrifuge plate for 15 minutes at 4500 rpm.
6. Place the plate into the sample manager or store at 4 ˚C for up to 5 days.

New sample preparation method employing a Phree phospholipid removal plate:

Serum was precipitated using ACN:Methanol (85:15) containing trideuterated 25-OH vitamin D_2 and D_3 internal standards. Sample precipitation, followed by vacuum filtration, was conducted on the Phree plate into a collection plate. A further aliquot of ACN wash was passed through the plate. The filtrate was analysed by reversed phase chromatography using a Phenomenex Kinetex core-shell 2.6 µm C18 100 Å 50 x 3 mm HPLC/UHPLC column on an ACQUITY UPLC separation module coupled to a Xevo TQS mass spectrometer (Waters Corp.). Details of the Phree protocol are:

1. Add 400 µL ACN with 0.1 % Formic acid and internal standard to each well of the Phree plate.
2. Add 100 µL of sample directly into the ACN with 0.1 % Formic acid. Heat seal any wells that are unused.
3. Vortex for 10 minutes at maximum speed taking care not to spill solvent.
4. Apply vacuum for 1-2 minutes and collect filtrate in a 96-well collection plate. The Phree sorbent selectively removes phospholipids from precipitated plasma while precipitated proteins are filtered out by the frit.
5. Add 500 µL of ACN with 0.1 % Formic acid to each well and vortex for 2 minutes at the maximum possible speed again taking care not to spill solvent.
6. Apply vacuum for 1-2 minutes and collect the filtrate into the same collection plate used in step 4, pooling the two fractions together.

continued

7. Drydown to completeness under a gentle stream of N_2.

8. Reconstitute in 500 µL ACN with 0.1 % Formic acid/0.1 mM Zinc sulfate (50:50).

LC/MS/MS Method

Column: Kinetex® 2.6 µm C18
Dimensions: 50 x 3.0 mm
Part No.: 00B-4462-Y0
Mobile Phase: A: 0.1541 g Ammonium acetate/1 mL Formic acid in 1 L of Deionized Water
B: 0.1541 g Ammonium acetate/1 mL Formic acid in 1 L of Methanol
Flow Rate: 0.6 mL/min
Gradient:

Time (min)	% B
0.0	82.5
1.8	82.5
2.0	99.5
3.5	99.5
4.0	82.5

Detection: MS/MS (Waters Corp. Xevo™ TQS)

Samples Used

96+ samples including: 4 calibrators, 5 QCs (3 UTAK and 2 Chromsystems (CS)) and the remainder - samples ranging from low (<15 nmol/L) to high (>180 nmol/L) concentrations.

Reference Range[5]

≤15 nmol/L – indicative of severe vitamin D deficiency

>15 nmol/L and ≤ 30 nmol/L – indicative of vitamin D deficiency

>30 nmol/L and ≤ 50 nmol/L – indicative of vitamin D insufficiency

>50 nmol/L – indicates adequate vitamin D status

>70 nmol/L – indicates optimal vitamin D status

>125 nmol/L – may indicate vitamin D toxicity

Results

Type	ID	Manuf. Conc. (nmol/L)	QC Range (nmol/L)	Original Protein Precipitaiton Method (nmol/L)	Phree™ Method (nmol/L)
Calibrator	Cal 0	2.8		N/A*	2.6
Calibrator	Cal 1	24		23.9	25.4
Calibrator	Cal 2	68.4		68.7	71.1
Calibrator	Cal 3	183		182.8	179.1
QC	UTAK1	25.3	21-29	28.2	25.6
QC	UTAK2	65.3	62-83	68.5	65.2
QC	UTAK3	193.1	155-210	193.2	179.4
QC	CS1	38.5	29.9-44.8	37.2	43
QC	CS2	139.7	107-160	122.3	126.7

* Value not obtained due to sample matrix interferences

Figure 2.
Extracted Calibrator 0 Chromatograms

Protein Precipitation

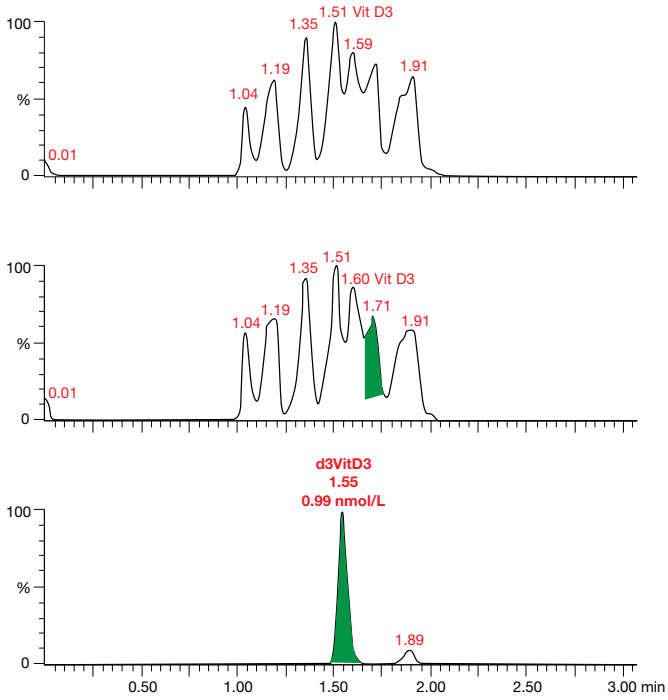


Figure 2. (cont.)
Extracted Calibrator 0 Chromatograms

Phree™ Phospholipid Removal

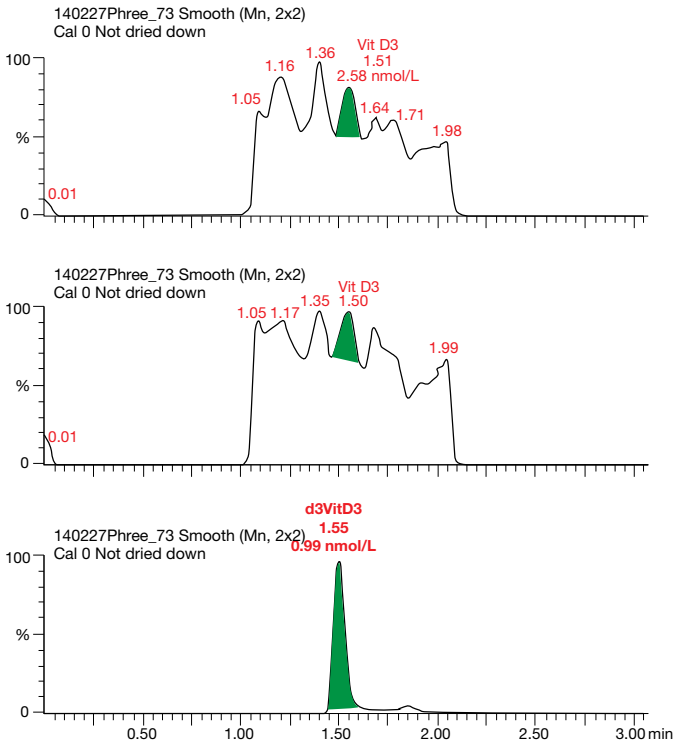


Figure 3.
Extracted Calibrator 1 Chromatograms

Protein Precipitation

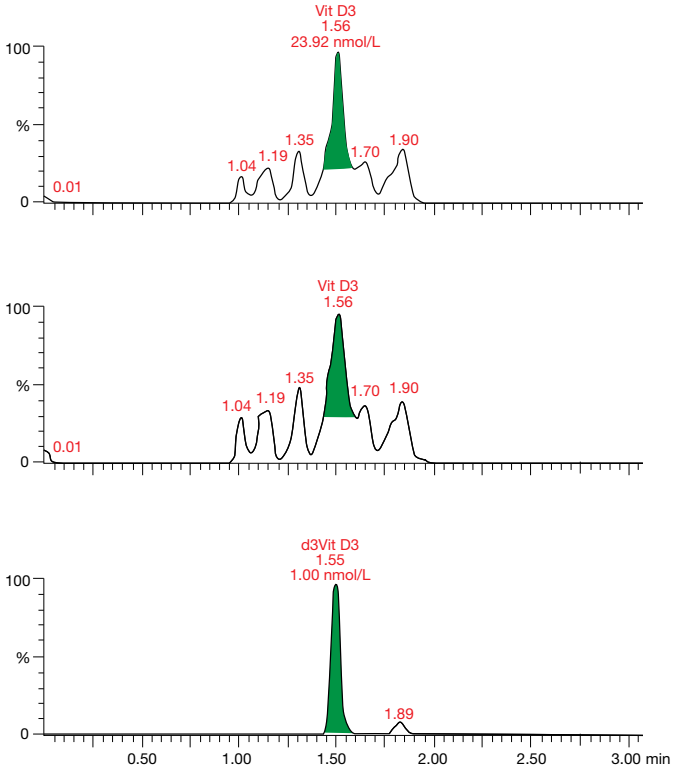


Figure 3. (cont.)
Extracted Calibrator 1 Chromatograms

Phree™ Phospholipid Removal

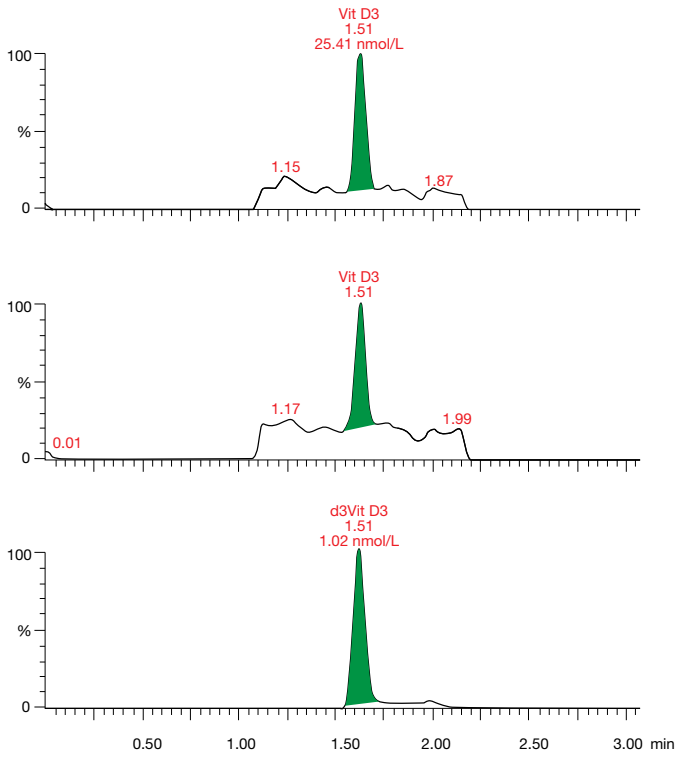


Figure 4.
Protein Precipitation Calibration Curve

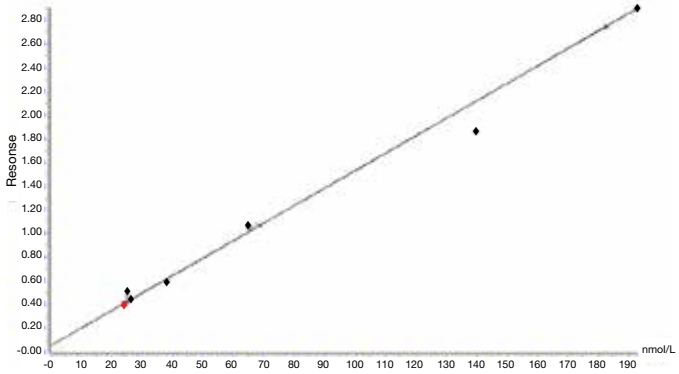


Figure 5.
Phree™ Phospholipid Removal Calibration Curve

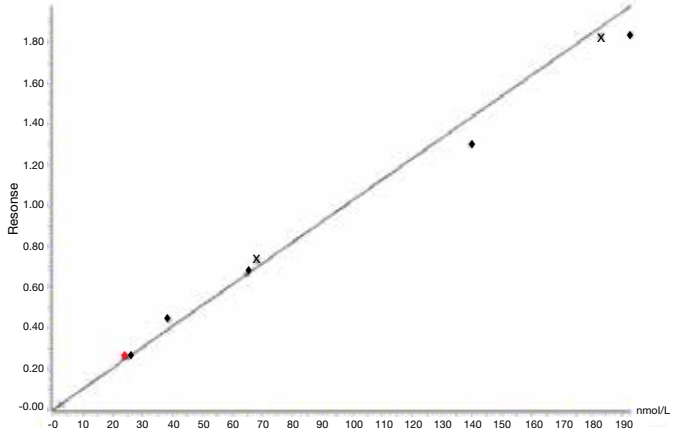


Figure 6.
Comparison of Phree™ Phospholipid Removal and Protein Precipitation Concentrations over a Range of Deficient Samples

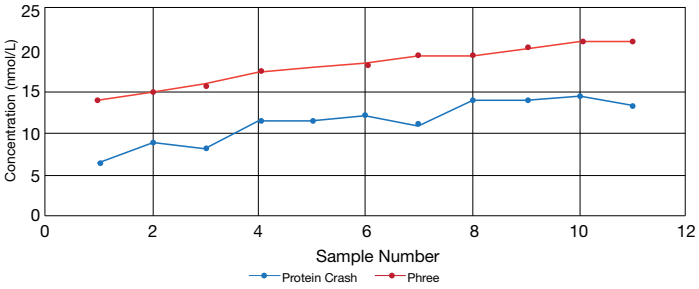


Figure 7.
Comparison of Phree Phospholipid Removal and Protein Precipitation Concentrations over a Large Range of Samples

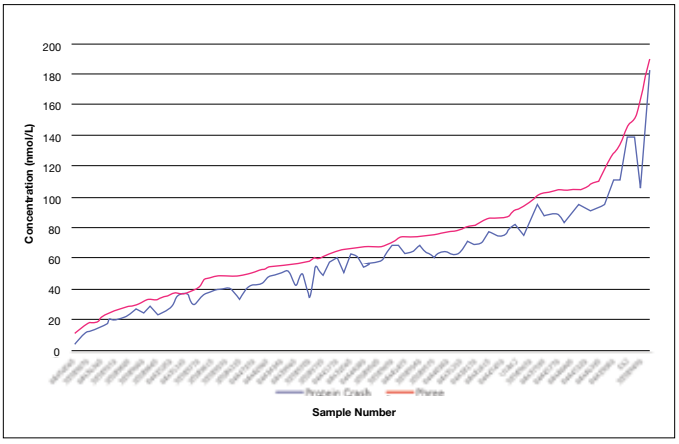
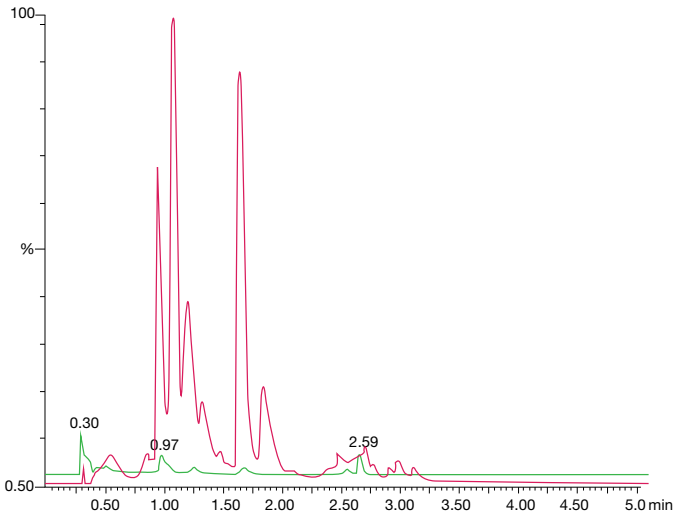
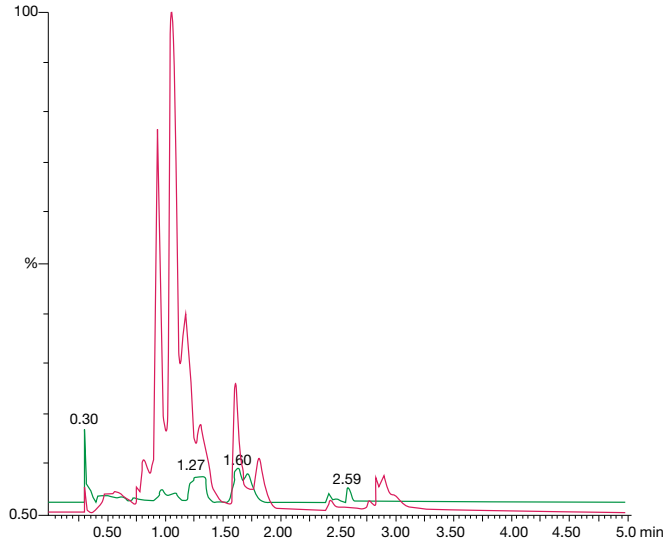


Figure 8.
Phospholipid Profile Overlays (m/z 184/184)

Cal 0 - Phospholipid profile



Sample - Phospholipid profile



Discussion

When looking at the calibration results presented in **Figure 1** it is evident that both the protein precipitation and Phree™ phospholipid removal methods gave very similar numbers to one another in terms of concentrations. Both were also within the manufacturer's acceptable concentration range. Importantly it was observed that due to matrix interferences, the 'Cal 0' calibrator was not detected after the original protein precipitation method. The extracted calibrator 0 chromatograms were further examined (**Figure 2**), the vitamin D_3 peak was not detected at 1.55 minutes (retention time of internal standard) using the protein precipitation method, although there were peaks on either side (1.51 and 1.60) which may have been the vitamin, however this is unlikely as normally the vitamin has the same retention time (RT) as its internal standard (IS). A vitamin D_3 peak was clearly visible at the retention time of the IS (1.51) using the Phree phospholipid removal method. Both the vitamin and its IS were at slightly shorter retention times compared to the original method. This was a result of the strength of the diluent. To confirm, a test was carried out to dilute the sample with water prior to injection and indeed this did increase the RT of the sample and IS.

Figure 3 shows the extracted 'Cal 1' results for ~25 nmol/L standards. The 25-OH vitamin D_3 peak at 1.55 minutes was detected at this level using the protein precipitation method, however there appeared to be variable ion suppression. This gives a positive bias to the peak area and affects quantitation. The peak resulting from the Phree extraction was clear of any visible ion suppression interferences and generally there were less peaks around the main peak. This led to a more accurate quantitation of the peak.

Comparing the calibration curves obtained by the protein precipitation method (**Figure 4**) and the Phree method (**Figure 5**) it can clearly be seen that there is a positive bias shown on the protein precipitation calibration curve. A lack of a Cal 0 value and a positive-biased Cal 1 area would have contributed to this. Due to the cleaner sample resulting from the Phree extraction protocol, no positive or negative intercept was observed. This is backed up by the values observed when the calibration curve was generated. Critically, the LLOQ calibrator was detected and closely matched the quoted concentration (**Figure 1**).

As a result of the cleaner extracts and more accurate calibration curve, quantitation of the vitamin from deficient samples showed higher values from the Phree extraction (**Figure 6**). All but one of these samples would have been re-classified as vitamin-deficient rather than severely deficient. This can also be seen in **Figure 7**, where for a full range of samples, the Phree method gave higher concentration values. Due to the wide acceptance range that the QC samples allow (**Figure 1**), it is difficult to say for certain which set of samples is more accurate. However, it can be assumed that samples which show a cleaner extract and calibrate with no bias probably have a greater chance of accuracy.

Finally the Phree extracts gave a 5-7 fold reduction in identified phospholipids both in the plasma samples and Cal 0 extracts (**Figure 8**). This reduction in phospholipids in the Phree extract would have reduced the amount of background 'noise' on the chromatograms allowing for better peak identification and quantitation. Moreover, it is known that phospholipids can cause analyte signal-suppression and in some circumstances can cause enhancement which can affect analyte quantitation[4]. In addition, fewer phospholipids will reduce contamination of HPLC columns and MS sources allowing for longer intervals between cleaning and hence reducing system downtime.

Conclusions

- Relative areas and thus concentrations of the plasma samples are universally higher from a Phree phospholipid removal extract.
- Detection and quantification down to the LLOQ was achieved using the Phree protocol, unlike the protein precipitation.
- Several severely deficient samples would have been classified as 'deficient' rather than 'severely deficient' if the Phree protocol had been used rather than protein precipitation.
- With a 5-7 fold reduction in phospholipid detected by the Phree method, columns and systems were both less contaminated.

References

1. Bilinski K, Boyages S., Evidence of overtesting for vitamin D in Australia: an analysis of 4.5 years of Medicare Benefits Schedule (MBS) data. BMJ Open (2013);3:e002955. doi:10.1136/bmjopen-2013-002955.
2. Carter G. D., Clinical Chemistry (2012) 58(3) 486–488.
3. Chromatography Today Helpdesk, Chromatography Today (2013) 6(4): 16-19.
4. Vitamin D and Bone Health: A Practical Clinical Guideline for Patient Management, National Osteoporosis Society
5. Maximising analyte recovery using the Phenomenex Phree SPE plate with an established LC-MS/MS method to quantify 25-OH Vitamin D2 and D3. Pruden, R., Dutton, J.J., Evans, L. Rudge, J. Clin Chem Lab Med 2014; 52(11):eA318-319.

Ordering Infomation

Phree™ Phospholipid Removal Products

Part No.	Description	Unit
8B-S133-TAK	Phree Phospholipid Removal 1 mL Tube	100/box
8E-S133-TGB	Phree Phospholipid Removal 96-Well Plates	2/box

Accessories

Collection Plates (deep well, polypropylene)		
AH0-7192	96-Well Collection Plate 350 µL/well	50/pk
AH0-7193	96-Well Collection Plate 1 mL/well	50/pk
AH0-7194	96-Well Collection Plate 2 mL/well	50/pk
AH0-8635	96-Well Collection Plate, 2 mL Square/Round-Conical	50/pk
AH0-8636	96-Well Collection Plate, 2 mL Round/Round, 8 mm	50/pk
AH0-7279	96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk
Sealing Mats		
AH0-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AH0-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AH0-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AH0-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AH0-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AH0-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AH0-7362	Sealing Tape Pad	10/pk
Vacuum Manifolds		
AH0-6023*	SPE 12-Position Vacuum Manifold Set, for tubes	ea
AH0-6024*	SPE 24-Position Vacuum Manifold Set, for tubes	ea
AH0-8950	96-Well Plate Manifold, Universal with Vacuum Gauge	ea

*Manifolds include: Vacuum-tight glass chamber, vacuum gauge assembly, polypropylene lid with gasket, male and female luers and yellow end plugs, stopcock valves, collection rack assemblies, polypropylene needles, lid support legs. Waste container included with 12-positive manifold.

Kinetex® Core-Shell HPLC/UHPLC Columns

5 µm Analytical Columns (mm)

Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard ULTRA Cartridges‡ 3/pk
C18	00B-4601-E0	00D-4601-E0	00F-4601-E0	00G-4601-E0	AJ0-8768 for 4.6 mm ID

5 µm MidBore™ Columns (mm)

Phases	50 x 3.0	100 x 3.0	SecurityGuard ULTRA Cartridges‡ 3/pk
C18	00B-4608-Y0	00D-4608-Y0	AJ0-8777 for 4.6 mm ID

5 µm Minibore Columns (mm)

Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard ULTRA Cartridges‡ 3/pk
C18	00A-4601-AN	00B-4601-AN	00D-4601-AN	00F-4601-AN	AJ0-8782 for 2.1 mm ID

2.6 µm Analytical Columns (mm)

Phases	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	SecurityGuard ULTRA Cartridges‡ 3/pk
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AJ0-8768 for 4.6 mm ID

2.6 µm MidBore™ Columns (mm)

Phases	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard ULTRA Cartridges‡ 3/pk
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775 for 3.0 mm ID

2.6 µm Minibore Columns (mm)

Phases	30 x 2.1	50 x 2.1	75 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard ULTRA Cartridges‡ 3/pk
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782 for 2.1 mm ID

1.7 µm MidBore Columns (mm)

Phases	50 x 3.0	100 x 3.0	SecurityGuard ULTRA Cartridges‡ 3/pk
C18	00B-4475-Y0	00D-4475-Y0	AJ0-8775 for 3.0 mm ID

1.7 µm Minibore Columns (mm)

Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard ULTRA Cartridges‡ 3/pk
C18	00A-4475-AN	00B-4475-AN	00D-4475-AN	00F-4475-AN	AJ0-8782 for 2.1 mm ID

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

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