ISOLUTE® PLD+

Protein and Phospholipid Removal Products – Questions and Answers

1. How do ISOLUTE® PLD+ Protein and Phospholipid Removal products improve my sample preparation?

ISOLUTE® PLD+ Protein and Phospholipid Removal plates and columns combine effective protein and phospholipid removal in a single product providing a very effective but extremely simple sample clean-up for LC-MS/MS analysis. Requiring next to no method development, ISOLUTE PLD+ can be integrated quickly and easily into routine workflow, increasing productivity and reducing instrument downtime. ISOLUTE PLD+ plates and columns remove more than 99% of plasma proteins and phospholipids, the main causes of ion suppression, leading to cleaner extracts and increased sensitivity (signal-to-noise (S/N)) for a broad range of analytes.

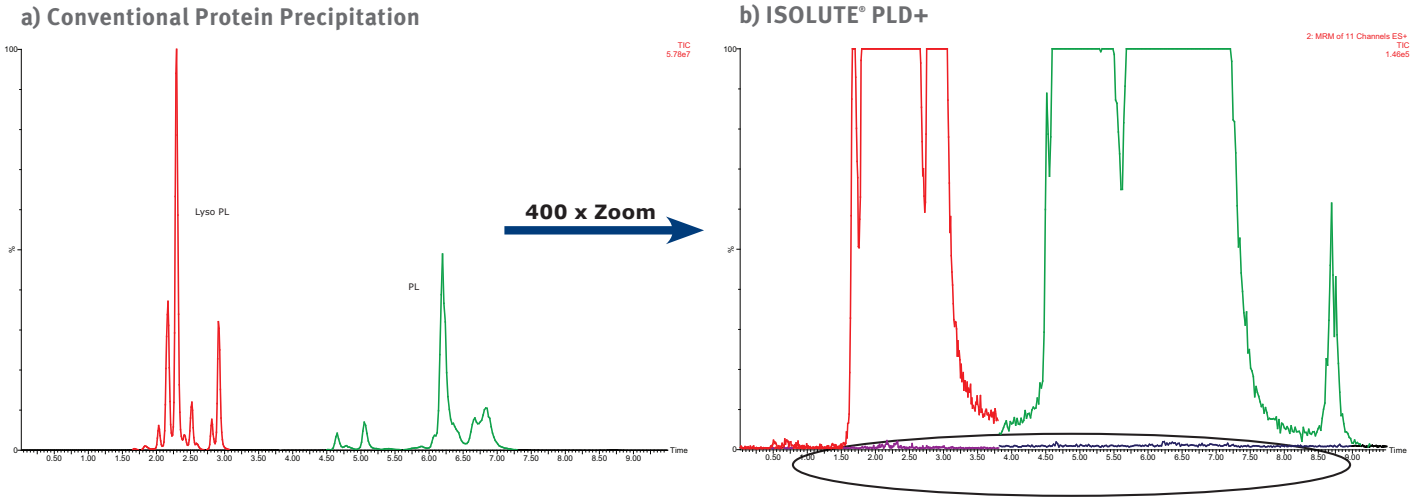


Figure 1. Phospholipids in human plasma treated by a) protein precipitation and b) ISOLUTE® PLD+. Lysophospholipids and phospholipids present in plasma treated by protein precipitation (red and green respectively) are shown in 1b on an expanded scale for direct comparison with residual levels of lysophospholipids and phospholipids present in ISOLUTE® PLD+ treated plasma (purple and blue respectively).

Biotage®

2. How do ISOLUTE® PLD+ products work?

In ISOLUTE® PLD+ plates and columns, optimized frit components are combined with a novel proprietary sorbent to provide simultaneous protein and phospholipid removal.

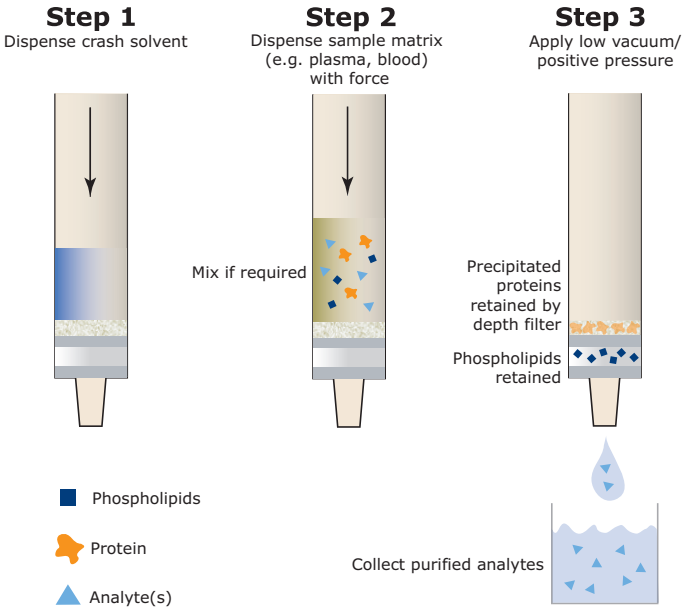


Figure 2. Typical ISOLUTE® PLD+ procedure.

The procedure is very simple

Step 1:

An appropriate volume of crash solvent is dispensed into the wells, and the proprietary treated top frit prevents solvent dripping. This allows for a more efficient 'solvent first' protein precipitation to occur when plasma samples are added.

Step 2:

An appropriate volume of plasma or other blood based matrix sample is added to the crash solvent. To ensure efficient protein precipitation, sample should be added directly into the crash solvent with force, and left for up to 5 minutes for protein precipitation to complete. A depth filter prevents precipitated proteins from blocking or plugging the wells or columns. Alternatively, ISOLUTE PLD+ plates and columns can be used with vortex mixing or aspirate/dispense protocols.

Step 3:

Low vacuum or positive pressure is applied, drawing the sample through the proprietary sorbent bed, for collection of purified extract in a pre-positioned collection vessel. The optimized bottom frit controls flow rates to ensure sufficient contact time for efficient phospholipid removal by the ISOLUTE PLD+ sorbent.

3. What is the ISOLUTE® PLD+ sorbent?

The ISOLUTE® PLD+ sorbent is a novel, proprietary multifunctional phase optimized to selectively retain phospholipids, but allow small molecule analytes (e.g. drugs and metabolites) with a broad range of chemical characteristics to pass through unretained. Unlike some competitor products, phospholipid retention is not based on a purely hydrophobic retention mechanism.

The combination of efficient protein and phospholipid removal with high analyte recoveries leads to better sensitivity than other commercially available phospholipid removal products.

4. What analytes can be extracted using ISOLUTE® PLD+ products?

ISOLUTE PLD+ plates and columns can be used to extract wide range of acidic, basic and neutral analytes, with widely differing polarity/hydrophobicity. In most cases, a single simple generic methodology can be used, virtually eliminating method development time.

Example:

Extraction of acidic, basic and neutral drugs from plasma using ISOLUTE PLD+ plates:

Procedure

1. Dispense acetonitrile (400 µL)
2. Dispense plasma sample (100 µL). Vortex mix for 30 s.
3. Apply vacuum (-0.2 bar) and collect purified extracts

After processing, samples were evaporated to dryness (Biotage® SPE Dry 96, 40 °C, 40 minutes), and reconstituted in mobile phase for analysis by LC-MS/MS.

Results

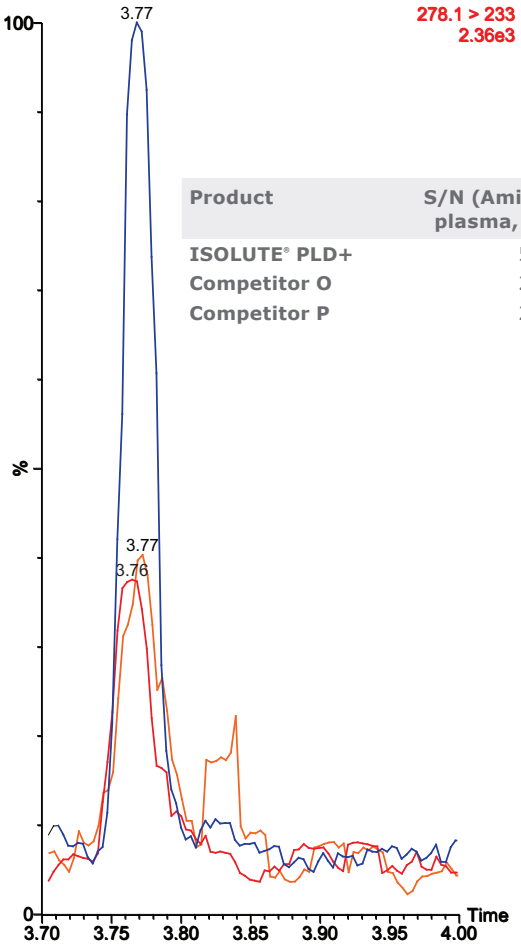
Table 1. Plasma spiked at 2 ng/µL.

Analyte	Functionality	pK*	logP*	% Recovery	% RSD (n=7)
Ketoprofen	Acidic	4.2	2.81	68	6.7
Sulindac	Acidic	4.0	3.59	75	4.1
Atenolol	Basic	9.1	0.16	74	5.8
Ranitidine	Basic	8.8	0.27	64	11.8
Procainamide	Basic	9.4	0.88	68	4.1
Salbutamol	Basic	9.4	1.31	74	6.2
Naltrexone	Basic	9.2	1.8	86	5.2
Metoprolol	Basic	10.8	1.88	77	4.8
Quinidine	Basic	9.28	2.88	75	5.0
Amitriptyline	Basic	9.4	3.1	76	4.5
Mianserin	Basic	8.3	3.6	67	2.2
Brompheniramine	Basic	9.2/3.6	4.06	61	3.0
Fluoxetine	Basic	9.5	4.2	83	3.5
Bretylium	Quaternary amine	N/A	-1.17	60	7.4
Acetaminophen	Neutral	N/A	0.34	83	3.7
p-toluamide	Neutral	N/A	1.18	80	5.6

*pK and logP values were obtained from literature or values were calculated if not available.

5. What detection limits should I expect?

This is highly dependent on the analyte and analytical system employed, but increased sensitivity can be achieved using ISOLUTE® PLD+ compared with competitor products. In this example, samples were spiked at a range of concentrations from 2 ng/mL to 20 pg/mL. At the lowest concentration level, S/N for some analytes was >50:1 (compared to ~ 20:1 using competitor products).



Product	S/N (Amitriptyline in plasma, 20 pg/mL)
ISOLUTE® PLD+	57:1
Competitor O	23:1
Competitor P	20:1

Figure 3. Relative peak intensity, 20 pg/mL amitriptyline in plasma (Blue = ISOLUTE® PLD+, Orange = Competitor O, Red= Competitor P).

6. Can I add sample matrix first rather than crash solvent first?

Yes, but we have found that a better protein precipitation (crash), and hence better sample purification, is achieved using the solvent first approach. The optimized top frit in ISOLUTE PLD+ can hold up acetonitrile for up to 24 hours, so there is no danger of the crash solvent dripping through before all samples have been loaded onto the plate. Automated systems such as the Biotage® Extrahera™ can utilize solvent or sample first methodologies using a forced crash procedure. This added simplicity can remove the necessity of offline mixing.

7. What crash solvent should I use with ISOLUTE® PLD+ products?

We recommend acetonitrile, but methanol can be used as an alternative. Addition of 1 % formic acid to the crash solvent can lead to more efficient protein precipitation, and may increase recovery of certain analytes.

Table 2. Recommended crash solvents.

Crash Solvent	Recommended Crash Solvent Volume: Sample Volume
Acetonitrile (or acidified acetonitrile)	300–400 µL (solvent): 100 µL (sample)
Methanol (or acidified methanol)	400 µL (solvent): 100 µL (sample)

For more viscous samples (e.g. whole blood), a crash solvent: sample of 4:1 (v/v) or higher may be required.

8. What vacuum or pressure levels should I use to process ISOLUTE® PLD+ plates and columns?

We recommend the use of the processing conditions shown in table 3 below.

Table 3. Recommended processing conditions.

Processing Method	ISOLUTE® PLD+ Plates	ISOLUTE® PLD+ Columns
Vacuum Manifold	-0.2 bar	-0.1 to -0.15 bar
Biotage® PRESSURE+ 96	3 psi	2 psi
Biotage® Extrahera™	Set to 0.3 to 0.4 bar	Set to 0.3 to 0.4 bar for 6 mins

Using these gentle processing conditions, processing of samples should be complete in 5 minutes or less.

Due to the optimized depth filter frit arrangement and narrow sorbent particle size range, ISOLUTE PLD+ products are less prone to plugging/blockage than competitor products, so do not require the high pressure/vacuum conditions recommended by other vendors. As a consequence ISOLUTE PLD+ plates and columns are easier to use than some competitor products on automation systems utilizing vacuum processing.

For particularly viscous samples, it may be necessary to increase processing pressure/vacuum. In this case, phospholipids will be removed, but removal efficiency may be reduced.

9. How much sample can I load?

Most commonly, 100 µL of sample is loaded into 300–400 µL of crash solvent. However, 200 µL sample matrix with 600–800 µL of crash solvent can be loaded without loss of performance.

10. What sample volume should I get back using ISOLUTE® PLD+ plates?

As with all protein precipitation based methods, a loss of sample volume can be expected. This can vary with sample type and the solvent used. Typically, ~ 75% of the original sample plus crash solvent volume is recovered following purification.

11. What if I don't have enough samples to utilize an entire 96-well plate?

You can process a partial ISOLUTE® PLD+ plate by sealing the unused wells with a piercable sealing cap (P/N 121-5204) or sealing tape. Alternatively, up to 96 individual ISOLUTE PLD+ tabless 1 mL columns can be processed using specially designed racks on Biotage (R) Pressure+96, Extrahera or VacMaster-96 systems, providing a flexible option for processing variable sample numbers.

12. What's the maximum total volume (sample + crash solvent) that I can load into each well of an ISOLUTE® PLD+ plate?

We would not recommend exceeding a total volume of ~1500 µL, to prevent well to well cross contamination on processing. If vortex mixing, this volume should be reduced to a maximum of ~1350 µL.

13. Can I purify samples other than plasma using ISOLUTE PLD+ products?

Yes, ISOLUTE PLD+ products can be used to remove phospholipids from other sample matrices, including serum, whole blood and tissue homogenates.

Special considerations for whole blood
It is particularly important to ensure an efficient crash to prevent breakthrough of red blood cells/extract coloration. Consider vortex mixing or aspirate/dispense cycles to improve crash efficiency.

Special considerations for tissue homogenates
Centrifugation of the sample prior to clean up on ISOLUTE PLD+ plates or columns may improve performance.

14. How do I maximize analyte recoveries?

- The addition of 1% (v/v) formic acid to the crash solvent may improve recoveries of protein bound analytes. Other acids (e.g. acetic) may also be used.
- A higher solvent to sample ratio (e.g. 5–10 parts solvent to 1 part sample) may also increase recovery of certain analytes. Refer also to Q 12.
- If analytes have low solubility in acetonitrile, evaluate the use of methanol or acidified methanol as crash solvent.
- Alternatively, evaluate dilution of sample with water before or after acetonitrile crash to improve analyte solubility.

15. What should I do if I get a cloudy extract?

A cloudy extract suggests an incomplete protein precipitation has occurred, due to insufficient mixing of the sample and crash solvent. To ensure good mixing, try the following practical tips.

- Add solvent first into each well or column not sample first, and ensure sample is applied with force directly into the centre
- Wait 5 mins for protein precipitation to occur
- Evaluate mixing by aspiration (2–3 times)
- Evaluate mixing by vortex, and increase intensity/time if required
- Consider increasing the ratio of crash solvent to sample.

16. What if my analyte or sample type or volume is incompatible with ISOLUTE® PLD+ purification?

If your analytes or sample size requirements are not amenable to clean up using ISOLUTE PLD+ plates or columns, we recommend evaluation of an alternative sample preparation technique such as supported liquid extraction (using ISOLUTE SLE+ plates or columns) or solid phase extraction (using EVOLUTE® EXPRESS SPE products). Contact Biotage for more details.

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