Sample Preparation using ISOLUTE® PPT+ Protein Precipitation Plates

Chemistry Data Sheet

This Chemistry Data Sheet describes the use of ISOLUTE® PPT+ Protein Precipitation Plates for the high throughput sample preparation of biological fluids (plasma, serum or blood).

Protein precipitation is a routinely used, high throughput sample preparation technique for removal of protein from biological fluid samples prior to analysis by LC-MS/MS.

Historically, protein precipitation has been carried out in vials or collection plates, followed by centrifugation; the supernatant is then transferred for analysis. Protein precipitation by filtration in the 96-well format has more recently been used as a high throughput, easy to automate alternative to the traditional centrifugation based technique. However, most filterplates require the plasma sample to be dispensed before the precipitating solvent is added (the 'plasma first' method), leading to leaking, cloudy filtrates and blocked wells.

ISOLUTE PPT+ Protein Precipitation Plates have been designed to overcome the problems of the first generation 96-well filterplates. The functionalized bottom frit holds up organic solvents, in particular acetonitrile, allowing the precipitating solvent to be dispensed into each well prior to sample addition. This 'solvent first' methodology is optimal for both high efficiency protein precipitation and automation, since the solvent first approach negates the need for vortex mixing.

The system has an optimized porosity distribution and acts as a depth filter, retaining the precipitated protein without well blockage.

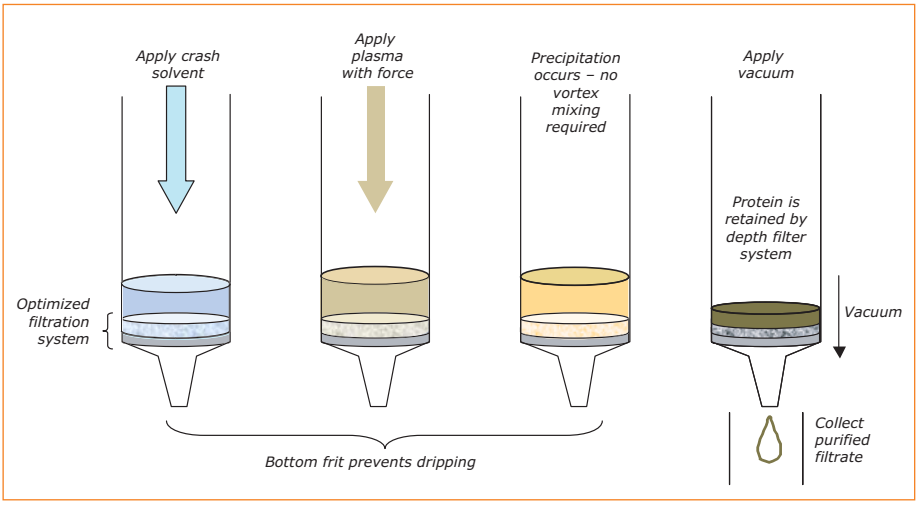
'Solvent First' Methodology for Protein Precipitation

ISOLUTE PPT+ plates can be used to process plasma sample volumes of up to 400 µL, using the optimal acetonitrile/plasma ratio of 3:1 (v/v).

The method below describes the procedure for processing **100 µL** plasma. For plasma volumes of less than 15 µL, refer to note 1 on page 2.

1. Place the ISOLUTE PPT+ plate onto a suitable 96-well sample processing manifold (e.g., a VacMaster™-96 Sample Processing Manifold). Ensure that a 96-well collection plate is positioned inside the manifold to collect the filtrate.
2. Dispense 300 µL of acetonitrile into each well. No solvent 'dripping' through the filterplate will occur.
3. Add 100 µL of plasma sample with force to each well. Again, no 'dripping' through the filterplate will occur. Allow the plate to stand for approximately 5 minutes. No vortex mixing is required.
4. Apply vacuum to filter the sample, and collect the purified filtrate. For the fixed well ISOLUTE PPT+ plate, application of –15 "Hg vacuum for 3 minutes is usually adequate for complete filtration. For the versatile ISOLUTE Array format a vacuum of –20 "Hg for 3 minutes is required.

Biotage



'Solvent First' Procedure using ISOLUTE PPT+ Protein Precipitation Plates

Notes

1. Using ISOLUTE PPT+ plates with the 'solvent first' methodology, the minimum effective volume of acetonitrile that should be used is 50 µL. This is suitable for precipitation of 15 µL plasma or less.

2. The maximum practicable volume of plasma that can be processed is 400 µL, using 1200 µL of acetonitrile (total volume per well is 1600 µL) in the fixed well format.

3. Acetonitrile has been found to be the most effective solvent for precipitating protein. Alternatively, acid precipitation may be used e.g. , 1 M trichloroacetic acid (TCA) in a 3:1 (v/v) ratio (TCA / plasma). The same dispensing method should be used for these alternative precipitating solvents, and no dripping will occur until vacuum is applied.

4. We recommend that internal standard is added to the plasma sample and not the precipitating solvent, so that binding of the analyte to plasma components can be better mimicked. As the filtration system holds up organic solvents, the sample will not drip though the plate until vacuum is applied.

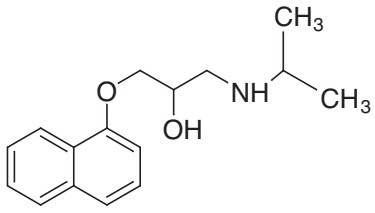
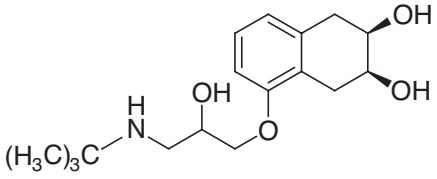
5. Serum or blood samples may also be processed, however, additional solvent may be required to fully precipitate the protein.

Experimental Data

1. Analyte Recovery

Using the 'solvent first' methodology as described, ISOLUTE PPT+ plates were used to isolate two probe compounds, propranolol and nadolol, from human plasma samples. Analyses were performed using HPLC-UV and LC-MS/MS respectively. An external standard was used for quantification. Following filtration, samples were evaporated at 40 °C, and reconstituted in 1 mL of mobile phase.

Analytical Conditions

Propranolol Concentration: 10 ng/100 µL plasma 		**Nadolol** Concentration: 10 ng/100 µL plasma 	
HPLC-UV Conditions		**HPLC and MS-MS Conditions**	
Column:	Genesis 4 µm C18, 4.6 x 150 mm	**Column:**	Polaris 3 µm C18-A, 50 x 2.0 mm
Mobile Phase:	0.2 % phosphoric acid pH 2.5 : MeOH (60:40, v/v) and 200 µL DEA	**Mobile Phase:**	0.1 % formic acid : MeCN : MeOH (70:25:5, v/v)
HPLC System:	Agilent HP1100	**HPLC System:**	Varian ProStar binary pump
Flow Rate:	1.4 mL/min	**Injection Volume:**	40 µL
Injection Volume:	40 µL	**MS System:**	Varian 1200L Triple quadrupole
Wavelength (λ):	220 nm	**Ionization Mode:**	ESI+
		SRM Transition:	310 > 254 (-16 eV)

Results

Analyte	% Recovery	% RSD
Propranolol	93.6	7.6
Nadolol	75.4	6.1

Conclusion

ISOLUTE PPT+ plates can be use to achieve high, reproducible recovery of analytes from plasma samples.

2. *Protein Removal*

Efficiency of protein removal using the 'solvent first' protein precipitation methodology was compared with the 'plasma first' method.

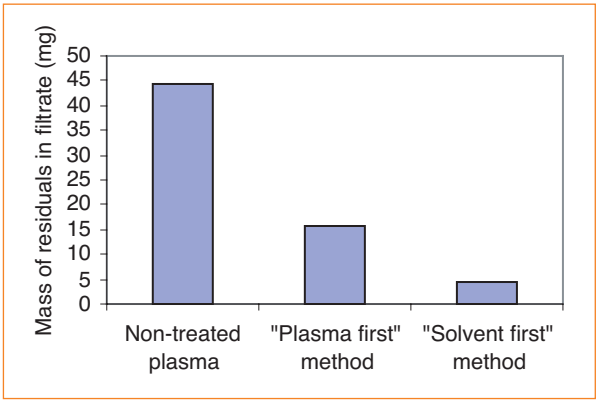
(i) Gravimetric Analysis of Residual Endogenous Material

Samples prepared using 'solvent first' (**page 1–2**) and 'plasma first'[1] methodology were compared to non-treated plasma. The amount of residual endogenous material remaining in the sample after filtration was determined gravimetrically.

For both methods, the filtrates from 5 wells were pooled together (total plasma volume 500 µL), and evaporated at 40 °C to constant weight. Non-treated plasma: 500 µL was dispensed into a pre-weighed vial and evaporated at 40 °C to constant weight.

Sample	Mass of residuals in filtrate	% Reduction
Non-treated plasma	44.0 mg	-
'Plasma first' method	15.7 mg	64 %
'Solvent first' method	4.4 mg	90 %

Results



Conclusion

The 'solvent first' method, using ISOLUTE PPT+ plates gives more efficient protein removal than the 'plasma first' method. Compared with non-treated plasma, ISOLUTE PPT+ plates reduce the amount of endogenous material (proteins, lipids, etc) in the prepared sample by up to 90%.

(ii) Effect on Ion Suppression

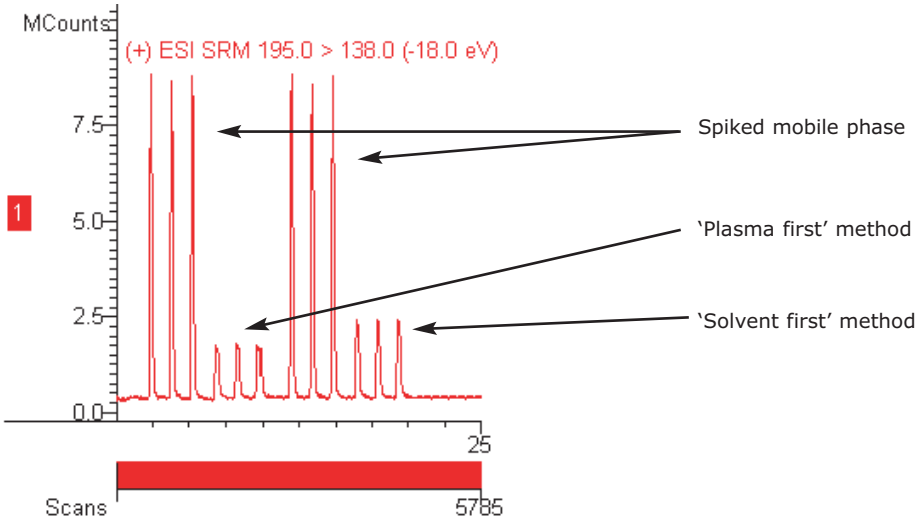
The effect of protein precipitation filtrates on analyte signal was investigated by flow injection analysis (FIA)[2] using electrospray LC-MS/MS.

100 µL blank human plasma was processed using either the 'plasma first' or 'solvent first' methods described previously. Blank plasma filtrates were evaporated and reconstituted with mobile phase containing caffeine at 1 µg/mL. 5 µL aliquots of the filtrates were injected directly into the ES interface without an HPLC column in place. Three replicate injections were analysed in SRM mode and compared mobile phase containing caffeine.

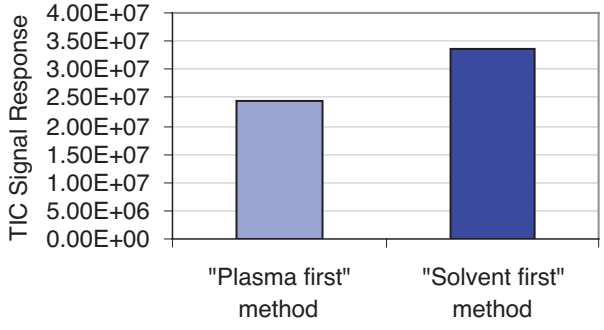
LC-MS/MS Conditions and Apparatus:

Mobile Phase:	MeCN : Water (50:50, v/v) containing 0.1% formic acid
HPLC System:	Varian ProStar binary pump / well plate sampler
MS System:	Varian 1200L Triple Quadrupole
Ionization Mode:	ESI+, SRM Transition: Caffeine 195 > 138

Results



Mass chromatogram showing effect of residual endogenous material from 'plasma first' and 'solvent first' filtrates on signal response of caffeine, compared to spiked mobile phase.



Conclusion

The 'solvent first' method gives reduced ion suppression compared to the 'plasma first' method. Signal intensity is 37 % higher using the 'solvent first' method with ISOLUTE PPT+ plates.

References

1. Chemistry Data Sheet TN120 Sample Preparation by Protein Precipitation using the ISOLUTE Protein Precipitation Plates, Biotage AB.
2. R. Bonfiglio, R.C. King, T.V. Olah, K. Mwerkle; *Rapid Commun. Mass Spectrom.*, (**1999**) *13* 1175-1185.

ISOLUTE PPT+ Product Ordering Information

Part Number	Description	Quantity
Fixed Well Plate		
120-2040-P01	ISOLUTE PPT+ fixed well plate, 2 mL	1
Versatile ISOLUTE Array Plate		
120-2040-R	ISOLUTE Array PPT+ wells, 1 mL	100
120-2040-T	ISOLUTE Array PPT+ wells, 2 mL	100
120-2040-RP	ISOLUTE Array PPT+ plate, 1 mL	1
120-2040-TP	ISOLUTE Array PPT+ plate, 2 mL	1
ISOLUTE Array Accessories		
120-1000-P01	ISOLUTE Array base plate	1
120-1200	Strip of 8 base plate sealing plugs*	50
120-1201	Luer adaptors (to fit any standard sample processing manifold)	25
120-1202	Well removing tool	1
*required when processing a partially populated EVOLUTE Array plate		
Collection Plates		
121-5201	Collection plate - 350 µL	50
121-5202	Collection plate - 1mL	50
121-5203	Collection plate - 2mL	50

For more information on the VacMaster-96 Sample Processing Manifold, please request Technical Note **PS408 – VacMaster-96 Sample Processing Manifold for 96-well Sample Preparation Applications.**

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