This Data Sheet Contains Important Information About The Product.

Instructions & Troubleshooting for HybridSPE®-Phospholipid (PL) 96-well Plates & Cartridges

Product Description

It is well-known that phospholipid contamination is one of the principal causes of ion-suppression when analyzing small molecules in biological matrixes via LC-MS-MS. Due to the inherent chemical nature of phospholipids (hydrophobic tail + zwitterionic polar head group), phospholipids are often co-extracted with analytes of interest during sample preparation and can be difficult to resolve during LC-MS analysis. This is especially true as shorter analytical LC-MS run times and ballistic gradients become increasingly mainstream.

HybridSPE-PL technology is a simple and generic sample prep platform designed for the gross level removal of endogenous protein and phospholipid interferences from biological plasma and serum prior to LC-MS or LC-MS-MS analysis. Biological plasma or serum is first subjected to protein precipitation via the addition and mixing of acidified acetonitrile. Precipitated proteins are then removed by centrifugation and the resulting supernatant is loaded on the HybridSPE 96-well plate or cartridge which acts as a chemical filter that specifically targets the removal of endogenous sample phospholipids. The phospholipid retention mechanism is based on a highly selective Lewis acid-base interaction between the proprietary zirconia ions functionally bonded to the HybridSPE stationary phase and the phosphonate moiety consistent with all phospholipids. The resulting eluent is ready for immediate LC-MS or LC-MS-MS analysis. There are two versions of the 96-well plate available for the HybridSPE-PL; the standard HybridSPE-PL is designed for use with biological sample volumes between 100-300 µL. The HybridSPE-PL Small Volume plate is designed for use with biological sample volumes between 20-40 µL.

An alternative "in-well precipitation" method is available for the HybridSPE 96-well version or ultra cartridge in which biological plasma/serum is first added to the 96-well plate or ultra cartridge followed by acidified acetonitrile (precipitation agent). After a brief mixing/vortexing step, vacuum is applied. Because the 96-well version or ultra cartridge contain a series of low porosity hydrophobic filters/frits, the packed-bed filter/frit assembly acts as a depth filter facilitating the concurrent removal of both phospholipids and precipitated proteins during the extraction process.

HybridSPE-PL Small Volume

"In-Well Precipitation" Method for HybridSPE-PL 96-well Plate or Ultra Cartridge

1. Spike biological plasma/serum sample and add I.S. as necessary.
2. Add the appropriate quantity of plasma/serum sample to the HybridSPE-PL plate or cartridge (Table 1) followed by a precipitating agent consisting of 1% formic acid in acetonitrile at a1:3 (v/v) ratio (do not apply vacuum at this time).

Table 1. HybridSPE-PL Sample and PPT Agent Guidelines

	Small Volume Plate	Standard Plate	Ultra Cartridge
Plasma/serum	20-40 µL	100-300 µL	30-100 µL
Precipitating agent	60-120 µL	300-900 µL	90-300 µL
Typical sample loss	30 µL	80 µL	90 µL

Note: Every effort should be made to minimize the distance between the collection plate and the bottom of the HybridSPE-PL plate to eliminate the potential for well-to-well cross-contamination. Shims or spacers may be required to raise the collection plate to the proper level in order to properly space the Hybrid plate and collection plate.

3. Perform in-well protein precipitation by agitating on an oscillating table and mix for 2 minutes at a setting of 1000 oscillations/minute. A 96-well plate sealing mat may be necessary to avoid potential spillage, cross-contamination, and /or spraying. Alternatively, protein precipitation can also be performed via robotic liquid handlers through repeated dispense and aspirations.
4. Transfer the HybridSPE-PL plate or ultra cartridge to a vacuum manifold, and apply vacuum (-10 to 15 in. Hg). Be sure to place a small volume collection device within the vacuum manifold prior to processing. **Note:** For optimum performance (phospholipid removal and analyte recovery) use 10" Hg (vacuum) for a minimum of 4 minutes to ensure samples have fully eluted.
5. Collect the resulting eluent and directly analyze via LC-MS. No further processing (evaporation/reconstitution) of the sample is necessary unless concentration of the eluent is desired prior to LC-MS analysis.

Note: The above "In-Well Precipitation" method is only recommended for 96-well or ultra cartridge-applications. The standard HybridSPE cartridges do not contain the necessary frits/filters to adequately remove precipitated proteins during extraction. Standard cartridge users should use the "Off-line Precipitation" Method described that follows.

"Off-line Precipitation" Method for 96-well or Cartridge Format:

1. Spike biological plasma/serum sample and add I.S. as necessary.
2. Transfer plasma/serum sample to the proper centrifugation tube or 96-deep well collection/reservoir plate and precipitate proteins by combining 100-300 µL plasma/serum sample with a precipitating agent consisting of 1% formic acid in acetonitrile (1:3, v/v). **Note:** consistent results have been obtained by combining 100 µL plasma/serum + 300 µL 1% formic acid in acetonitrile.
3. Facilitate precipitation by agitating/vortexing for 1-3 minutes; and remove precipitated protein by centrifugation (3000 rpm for 2-5 minutes).
4. Transfer the resulting supernatant or an aliquot of the resultant supernatant to the HybridSPE 96-well plate or cartridge. Using a vacuum manifold, apply vacuum (-10 to -15 in. Hg). **Note:** it should take 1-2 minutes for the precipitated sample to completely pass through the packed bed; however, more time may be necessary for highly viscous samples.

   Collect the resulting eluent and directly analyze via LC-MS or LC-MS-MS. No further processing (evaporation/reconstitution) of the SPE eluent is necessary unless eluent concentration is desired prior to LC-MS analysis.

Featured Products

Description	Pkg. Qty.	Cat. No.
HybridSPE-PL 96-well Plate, 50 mg/well	1	575656-U
	20	575657-U
HybridSPE-PL Cartridge, 30 mg/1 mL	100	55261-U
	200	55276-U
HybridSPE-PL, Small Vol. 96-well plate, 15 mg/well	1	52794-U
	20	52798-U
HybridSPE-PL Cartridge, 500 mg/6 mL	30	55267-U
HybridSPE-PL Ultra Cartridge, 30 mg/1 mL	100	55269-U

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Troubleshooting & Frequently Asked Questions

1. **Can I use HybridSPE-PL with smaller plasma volumes (e.g., 20-40 µL plasma)?**

   Yes, the HybridSPE-PL Small Volume is designed for use of plasma/serum volumes between 20-40 µL. Larger sample volumes of 100-300 µL should be used on the standard HybridSPE-PL plate.

2. **Why is acetonitrile and formic acid used as a precipitating agent in the HybridSPE-PL method?**

   Acetonitrile is a commonly used protein precipitation agent when prepping plasma samples for LC-MS analysis. The resulting precipitated protein is easily filtered using the "In-well Precipitation" method and forms protein pellets easily when centrifugation ("Off-Line Precipitation" method) is preferred.

   The addition of 1-2% formic acid to the acetonitrile precipitating agent is critical because: 1) formic acid is a stronger Lewis base than most carboxyl (-COOH) groups found in acidic pharmaceutical compounds (inhibiting analyte retention on the HybridSPE phase) but not as strong a Lewis base as the phosphate moiety found in phospholipids; and 2) the low pH environment neutralizes residual silanol activity on the silica surface thereby eliminating secondary cation-exchange interaction with basic compounds of interest.

3. **What if my analyte(s) of interest are not soluble in acetonitrile?**

   Although some analytes may not be soluble in acetonitrile, after protein precipitation, the HybridSPE eluent will consist of 75% acetonitrile (w/ formic acid) and 25% aqueous (from the biological sample). The aqueous content of the sample should provide adequate solubility prior to LC-MS analysis.

   Alternatively, 1% ammonium formate in methanol may be used in place of 1% formic acid in acetonitrile. Ammonium formate in methanol provides increased solubility of polar compounds and precipitates proteins as well as acetonitrile allowing for both "Off-Line" and "In-well" precipitation methods.

4. **Can I increase assay sensitivity by either increasing sample volume and/or concentrating (evaporation and reconstitution) of the HybridSPE eluent?**

   It is not recommended applying >40 µL of biological sample to the HybridSPE-PL Small Volume plate, however biological sample volumes of >300 µL can be applied to the standard HybridSPE-PL plate. Some phospholipid breakthrough may occur with analytes of interest. 98-100% of biological phospholipids are removed when ≤300 µL plasma is applied to the standard HybridSPE phase. When increasing sample volume be sure to increase the volume of the precipitating agent accordingly. A 1:3 (v/v) plasma:precipitating agent ratio is necessary for optimal performance.

   Another strategy for increasing sensitivity is through evaporation of the HybridSPE eluent followed by reconstitution in a smaller volume of LC-MS mobile phase. The acetonitrile portion of the HybridSPE eluent greatly aids the evaporation process. On average it takes less than 10 minutes to evaporate 300-400 µL of HybridSPE eluent under nitrogen at 37 °C.

5. **Why is ion-suppression still evident during LC-MS analysis after HybridSPE-PL?**

   HybridSPE technology will only remove phospholipids and gross levels of precipitated protein from biological samples. Other chemical entities common to biological samples can lead to ion-suppression if not removed prior to LC-MS-MS analysis. It is important to identify the ion-suppression causing component to facilitate troubleshooting. It may be necessary to adjust chromatographic conditions to separate analytes of interest from interfering matrix components. Examples of non-phospholipid chemicals that can lead to ion-suppression include:

   - sodium citrate which is an anti-coagulant used to prepare plasma from blood
   - phthalates, plasticizers and other mold release agents found in plastic ware
   - polyethylene glycol which is a common dosing vehicle for many drugs
   - extractables from o-rings, plastic ware, and seals used to store biological samples

6. **Why is the resulting HybridSPE-PL eluent lower in volume than what was applied to the HybridSPE packed bed?**
   What are the effects of conditioning the phase?

   The dead volume for the standard HybridSPE packed bed is ~80 µL. The dead volume for the HybridSPE-PL Small Volume is ~30 µL, also, there is an evaporation effect on the eluent when using a vacuum manifold. When applying -15 in. Hg to the HybridSPE plate for 3 min. (time taken to pass the sample through the well plate), 10-20 µL of the volume of the SPE eluent can be lost due to evaporation during processing. Therefore, when processing a 400 µL precipitated sample (100 µL plasma + 300 µL precipitating agent) through the unconditioned HybridSPE phase, the resulting eluent will be a volume of ~ 300 µL.

   Although the volume is reduced during SPE processing, the final analyte concentration of the eluent does not appear to be affected. Nevertheless, addition of an I.S. is recommended prior to HybridSPE processing (which is standard for most sample prep techniques).

   If the analyst chooses to condition the HybridSPE phase with ≥80 µL of solution prior to sample addition, there could be a dilution effect. The final eluent volume will be ~80 µL greater than it should be. As a result, absolute recovery will appear lower than it actually is. If increasing signal response is necessary during LC-MS analysis, we recommend evaporating the eluent and reconstituting in a smaller known volume of LC mobile phase prior to LC-MS analysis.

7. **Why am I experiencing absolute recovery values > 100%?**

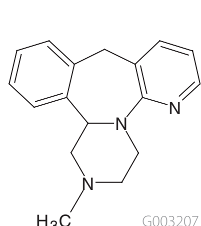
   The resulting HybridSPE eluate contains acetonitrile (volatile solvent). After the precipitated sample completely passes through the HybridSPE packed bed, the vacuum should be disengaged immediately. Further vacuum application can evaporate the eluate thereby erroneously giving misleadingly high analyte responses during subsequent LC-MS analysis.

8. **Why am I experiencing low absolute recovery of ≤50%?**

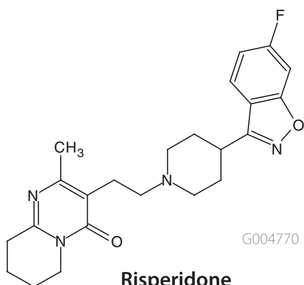
   The primary "In-well Precipitation" and "Off-Line Precipitation" procedures using formic acid and acetonitrile as a precipitating agent will work well for ~80% of the applications encountered. However, ~ 20% of the analytes will co-retain with phospholipids under these conditions resulting in absolute recoveries of ≤50%. On the next page, strategies are described on how to deal with low recovery compounds.

Secondary Procedure for Low Recovery Basic Compounds (contains amine functional groups):

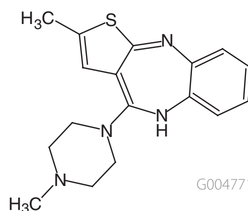
- Some basic compounds may experience low recovery when employing the primary method (1:3 plasma:1% formic acid in acetonitrile). Example compounds include:



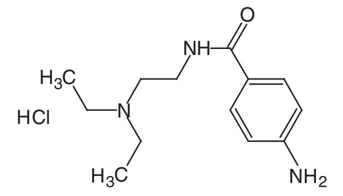
Mirtazapine



Risperidone



Olanzapine



Procainamide

- Low recovery of such basic compounds are caused by: 1) secondary weak cation exchange interactions between HybridSPE silanol groups (Si-O⁻); and 2) secondary HILIC interactions between HybridSPE silica surface and analytes

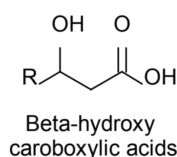
- We recommend combining: 1:3 plasma:1% ammonium formate in methanol followed by HybridSPE-PL processing as described in the standard recommended procedure(s).

Note:
- Recovery of basic and neutral compounds can improve from < 40% to > 89%
- NH_4^+ (ammonium formate) is a stronger counter-ion than H^+ (formic acid) inhibiting most basic compounds from interacting with HybridSPE silanol groups (Si-O⁻).
- Methanol is a more polar solvent than acetonitrile further inhibiting any potential secondary HILIC interactions between the analyte and HybridSPE silica surface.
- Note that ammonium formate in methanol is an excellent protein precipitation agent making the reagent amenable to both "Off-line" and "In-well" precipitation methods.

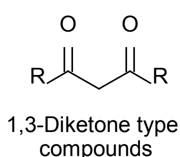
Secondary Procedure for Low Recovery Acidic Chelator & Chelator Compounds:

- Some acidic chelator and chelator compounds may experience low recovery when employing the primary method (1:3 plasma:1% formic acid in acetonitrile).

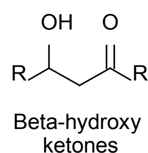
Chelation functional groups that can lead to low HybridSPE-PL recovery:



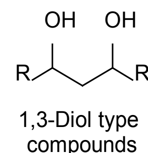
Beta-hydroxy caroboxylic acids



1,3-Diketone type compounds

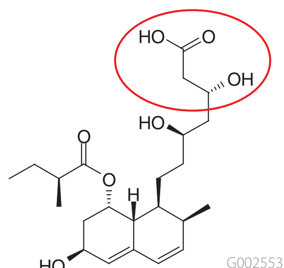


Beta-hydroxy ketones

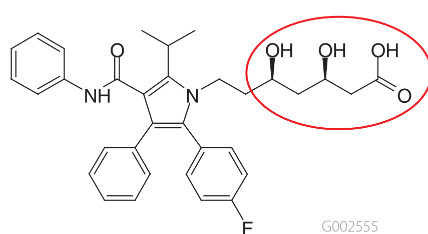


1,3-Diol type compounds

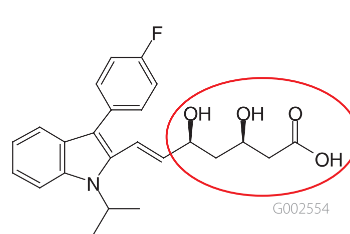
Example compounds with chelation functional groups:



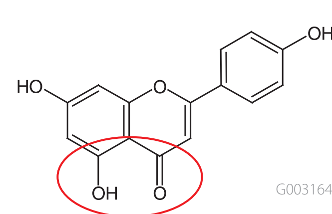
Pravastatin



Atonvastatin



Fluvastatin



Apigenin

- In such cases, using citric acid (a stronger Lewis base than formic acid) in acetonitrile as the precipitating agent will inhibit analyte retention while still allowing phospholipids to retain (be removed).

- When experiencing low recovery for such compounds, we recommend to first condition the HybridSPE phase with 400 µL 0.5% citric acid in acetonitrile (until flow has ceased). Combine 1:3 plasma:0.5% citric acid in acetonitrile followed by HybridSPE-PL processing on the conditioned phase using either the "Off-line" or "In-well" precipitation method.

Note:
- Recovery of chelator compounds can improve from < 40% to 65-95%
- Citric acid is a stronger Lewis base than formic acid inhibiting the retention of chelator compounds.
- Citric acid is not a strong enough Lewis base to inhibit phosphates (phospholipids) from retaining on the HybridSPE phase.

Related Products

Description	Pkg. Qty.	Cat. No.
96-well Protein Precipitation Filter Plate	1	55263-U
Supelco PlatePrep Vacuum Manifold	1	57192-U
96 Square/Deep Well Collection Plates, 0.35 mL, PP	50	575651-U
96 Square/Deep Well Collection Plates, 1 mL, PP	50	575652-U
96 Square/Deep Well Collection Plates, 2 mL, PP	50	575653-U
96 Square Well Pierceable Cap Mats	25	575655-U

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