# Instructions & Troubleshooting for HybridSPE®-Phospholipid Ultra Cartridge

## Product Description

The HybridSPE-Phospholipid Ultra Cartridge can clean up biological samples in three different ways, (1) remove small particles/debris so that the sample is suitable for Ultra High Pressure Liquid Chromatography (UHPLC) system, (2) remove protein by in-tube precipitation and (3) remove phospholipids by chemical adsorption/absorption.

It is well-known that phospholipid contamination is one of the principal causes of ion-suppression when analyzing small molecules in biological matrices by 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-MS analysis.

HybridSPE 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. 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.

## Generic Protocol for in-Tube Sample Preparation

"A typical ratio of protein crash solvent to plasma is 3:1. The following protocol is ideal for 100 µL of plasma. We have found that this tube can effectively remove phospholipids from a maximum of 100 µL of plasma.

1. Dispense 100 µL of plasma in each tube (add internal standard if necessary.)
2. Add 300 µL of crash solvent (e.g. acetonitrile with 1% formic acid).
3. Vortex for one minute at 4000 cycles/min. Be careful while vortexing multiple tubes at the same time, because it is critical that each tube be sufficiently mixed. If sample appears cloudy, mixing was not adequate.
4. Apply vacuum at -15 in Hg for two minutes or until the sample has passed completely through the sorbent bed. Another alternative is to apply positive pressure with a syringe attached to an adapter.

**Table 1. Properties of HybridSPE-Phospholipid Ultra Cartridge**

| Variable | Value |
|---|---|
| Hold up/dead volume (cartridge + media + frits + hardware + test tube) | Average 90 µL |
| Phospholipid removal capacity (plasma volume) | 100 µL maximum |
| Recommended plasma volume | 30 µL – 100 µL |

## Troubleshooting & Frequently Asked Questions

1. **Why is acetonitrile and formic acid used as a precipitating agent in the HybridSPE-Phospholipid Ultra Cartridge 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-tube Precipitation" method and forms protein pellets easily. The addition of 1-2% formic acid to 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.

2. **What if my analytes 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 replacing 1% formic acid in acetonitrile. Ammonium formate in methanol provides increased solubility of polar compounds and precipitates proteins.

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

   It is not recommended applying >100 µL of biological sample to the HybridSPE-Phospholipid Ultra Cartridge. 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 purge at 37 °C.

4. **Why is ion-suppression still evident during LC-MS analysis after HybridSPE-Phospholipid Ultra Cartridge?**

   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, plasticware, and seals used to store biological samples

5. **Why is the resulting HybridSPE-Phospholipid Ultra Cartridge 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 HybridSPE-Phospholipid Ultra Cartridge packed bed is ~90 µL. There is an evaporation effect on the eluent when using a vacuum manifold. When applying -15 in Hg to the HybridSPE plate for 2-3 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-Phospholipid Ultra Cartridge processing (which is standard for most sample prep techniques).

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If the analyst chooses to condition the HybridSPE-Phospholipid Ultra Cartridge with >90 µL of solution prior to sample addition, there could be a dilution effect. The final eluent volume will be ~90 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.

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

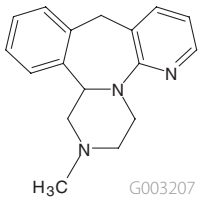
   The resulting HybridSPE-Phospholipid Ultra Cartridge eluate contains acetonitrile (volatile solvent). After the precipitated sample completely passes through the HybridSPE packed bed, the vacuum should be disengaged immediately. Further vacuum application can evaporate the eluate thereby erroneously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

7. **Why am I experiencing low absolute recovery of < 50%?**

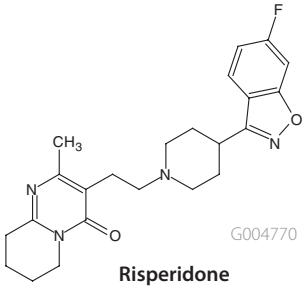
   The primary "In-Tube Precipitation" and "Off-Line Precipitation" procedures using formic acid and acetonitrile as a precipitating agent will work well for ~80% of the applications encountered. However, ~20% of the analytes will co-retain with phospholipids under these conditions resulting in absolute 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):**

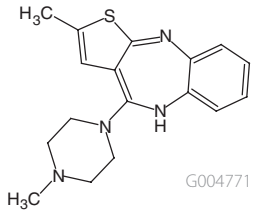
Some basic compounds may experience low recovery when employing the primary method (1:3 plasma:1% formic acid in acetonitrile). Example compounds include:



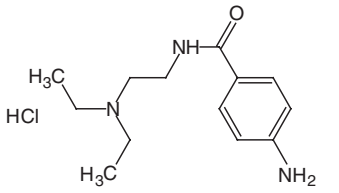

**Mirtazapine**




**Risperidone**




**Olanzapine**




**Procainamide**

Low recovery of such basic compounds are caused by: 1) secondary weak cation exchange interactions between HybridSPE silanol groups ($Si\text{-}O^-$); 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-Phospholipid processing as described in the standard recommended procedure(s).

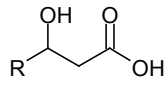
**Note:**

- 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 ($Si\text{-}O^-$).
- 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.

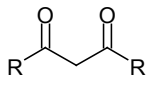
**Secondary Procedure for Low Recovery Acidic Chelator & Chelator Compounds:**

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

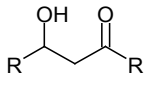
**Chelation functional groups that can lead to low HybridSPE-Phospholipid recovery:**



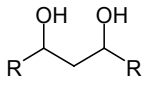
Beta-hydroxy caroboxylic acids



1,3-Diketone type compounds

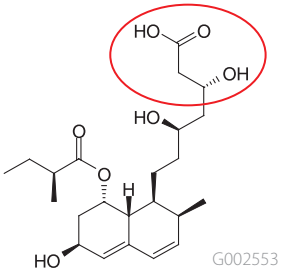


Beta-hydroxy ketones

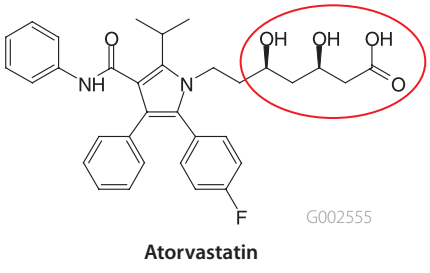


1,3-Diol type compounds

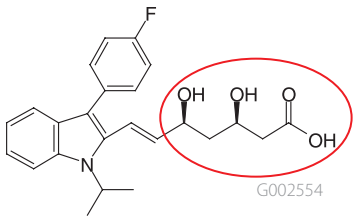
**Example compounds with chelation functional groups:**



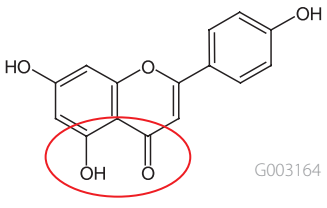

**Pravastatin**




**Atorvastatin**




**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-Phospholipid 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.

### Featured Products

| Description | Pkg. Size | Cat. No. |
|---|---|---|
| HybridSPE-Phospholipid Ultra Cartridge, 30 mg/1 mL | 100 | **55269-U** |
| HybridSPE-Precipitation 96-well Plate, 50 mg/well | 1 | **575656-U** |
| HybridSPE-Precipitation Cartridge, 30 mg/1 mL | 100 | **55261-U** |
| HybridSPE-Phospholipid Small Volume 96-well plate, 15 mg/well | 1 | **52794-U** |
| HybridSPE-Precipitation Cartridge, 500 mg/6 mL | 30 | **55267-U** |

### Related Products

| Description | Pkg. Size | 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** |