Troubleshooting Tips

Problem	Possible Causes and Solutions
No/low peptide recovery **Poor peptide recovery**	**1.** In most cases, solutions made within the protocol are not formulated and/or stored properly. It is recommended to use pre-made solutions, found at protifi.com. **2.** The applied protein sample did not contain sufficient SDS (5%). SDS is necessary for efficient capture in the protein-trapping matrix. High concentrations of SDS (up to 20%) do not affect S-Trap™ performance. Dilute samples can be concentrated on a SpeedVac or lyophilizer. **3.** It is important to use sodium DS not lithium; binding is sensitive to the counter ion. **4.** The applied protein sample was not acidified properly with phosphoric acid. This step is necessary for efficient protein denaturation and capture. Make sure the SDS-solubilized protein sample is acidified to a final concentration of 2.75% phosphoric acid and is highly acidic (pH ≤ 1); if needed, add additional phosphoric acid to reach pH ≤ 1. Phosphoric acid must be stored tightly capped as it will degrade upon atmospheric exposure. Old phosphoric acid has been found to be the cause of poor binding on multiple occasions. **5.** If the acidified SDS lysate/MeOH buffer solution was centrifuged before addition into the unit, it is possible the protein was pelleted out and did not enter the unit. All protein, including any particulate or colloid, must be transferred into the S-Trap™ unit. **6.** Unsheared DNA, highly viscous proteins (e.g. from mucosal tissues), or spin column overloading may necessitate significantly longer spin times. After each centrifugation step, make sure that all added solution has gone through the S-Trap™ column. Centrifuge longer as needed. Load the column multiple times until the full volume has been bound, removing waste as needed. **7.** The size of S-Trap™ unit should be matched to the amount of protein to digest (e.g. S-Trap™ micros are not recommended for > 100 µg; use S-Trap™ minis). MS-compatible detergents have been found to aid in recovery of low protein levels. Additional organic elutions may also aid in recovery. **8.** If the digest has dried on the column (for example, if the cap was not applied during incubation, or the column was forgotten), the protein-trapping matrix will need to be rehydrated to solubilize the peptides. Add Digestion Solution and let sit for 30 minutes, then centrifuge out. Repeat the solubilization and elution steps again. Additional elutions may assist in peptide recovery. Concentrate by lyophilization. **9.** The protein-trapping matrix retains proteins but not peptides. If digestion is incomplete, poor peptide recovery will result. Digestion can be monitored by gel as well as MS analyses of the flow through, washes, and elutions. **10.** Protease concentration may need to be optimized depending on sample and protease. Try different w/w concentrations of protease; different digestion temperatures; include a MS-compatible detergent like Rapigest™ or ProteaseMAX™ (should not be used at elevated digestion temperatures due to accelerated autolysis); and/or increase digestion time. Due to evaporation, digestion volume will likely need to be increased with digestion times longer than 1 hour. Do not apply < 1 µg of trypsin per S-Trap™ for efficient digestion or significantly increase incubation time; other proteases may require different minimum amounts. Trypsin/Lys-C mixes work better than trypsin alone. See Appendix C. **11.** Heating of the S-Trap™ may be insufficient during digestion. Ensure entire S-Trap™ column is exposed to heat and do not have the S-Trap™ column e.g. sticking out of the top of a heat block where it will receive insufficient heat. **12.** Applied protein sample is in the insoluble pellet. Insoluble sample can be processed as solids atop a separate S-Trap™ column. Perform all steps on the solid sample and homogenize the insoluble sample as much as possible before sample processing.

Problem	Possible Causes and Solutions
Solution substitutions needed for TEAB	**1.** Tris-HCl can be used in place of TEAB at the same concentrations and pH value for Binding/Wash Solution and Digestion Solution. Tris is not compatible with iTRAQ, TMT, or other amine labeling chemistries. **2.** Ammonium bicarbonate cannot be used in the Binding/Wash Solution due to insufficient solubility in 90% MeOH. Ammonium bicarbonate can be used in place of TEAB in the Digestion Solution.
Sample is viscous	**1.** PIXUL ultrasonication: Ultrasonicate for 5 minutes using 50 N pulses at 1 kHz with a 20 Hz burst rate. **2.** Covaris ultrasonication: On a S220, ultrasonicate for 6 minutes using 200 cycles per burst at 175 W and a 10% duty factor at 20 °C. **3.** Probe or horn sonicator: Settings depend on the power of the sonicator. Test power setting and length of sonication with a 1.5 mg/mL solution of salmon sperm DNA, sonicating until the viscosity is undistinguishable from an aqueous solution. **4.** Benzonase: Add $MgCl_2$ to Lysis Solution to yield a final concentration of 2 mM. Add 100 units of Benzonase per 50 µg of protein and incubate at RT for 5 minutes.
Contaminants in sample	**1.** Additional wash(es) may be performed and should be performed if contamination is observed by MS. You cannot over wash mammalian proteins with Binding/Wash Solution. **2.** For hydrophobic contaminants, such as lipids and wax; bind protein, wash 3 times with 50% $CHCl_3$/50% MeOH, filling the entire column each time, then perform 3 washes with standard methanolic Binding/Wash Solution.
Equipment differences for digestion	**1.** Preload dry incubators with beakers of water to saturate the air with water vapor and limit evaporation; let the beakers come to temperature before using the incubator or fill with hot water. **2.** If using a thermomixer, fill unused wells with water and cap. If samples appear to be drying out despite a water-saturated atmosphere, add additional Digestion Solution to compensate for evaporative loss.
Precipitate present when resuspending eluted peptides	Extended incubation in basic conditions (pH > 8.5) can partially solubilize the silica matrix; this is insoluble at low pH. To prevent formation, ensure the pH is 8.0 for 2 - 4 hour digests and between 7.5 and 8.0 for overnight digests. If precipitation is present, remove it by centrifugation, filtration, or high-pH C18 desalting; samples are then ready for MS analysis.