S-Trap™ Micro Universal MS Sample Prep Kit: Quick Card

① **1)** Lyse cells/dissolve ≤ 100 µg protein with **buffer 1**, 2X SDS solubilization buffer (white cap vial). If sample is liquid, add 11 µL 2X buffer 1 to 11 µL of sample. If sample is solid (cells or precipitated protein), dilute 2X buffer 1 to 1X with MilliQ water and add 22 µL. Dissolve and disrupt DNA by sonication or use Benzonase.

2) Quantify protein concentration by BCA. S-Trap™ Micros optimally digest a maximum of 100 µg.

② **3)** Add 1 µL of **buffer 2**, reducing reagent, red cap. Incubate 10 min at 55 °C.

③ **4)** Add 2 µL of **buffer 3**, capping reagent (yellow cap). Incubate at room temperature for 10 min.

5) If needed, clarify by centrifugation (20,000 g for 10 min); keep supernatant.

④ **6)** Add 2.5 µL **buffer 4** (green cap) to the protein sample.

⑤ **7)** Add 165 µL of **buffer 5**, S-Trap binding buffer (clear capped large bottles) to the acidified lysate. **Ensure 8.1 mL of HPLC grade methanol have been added to each bottle before use.**

8) Add mix into the S-Trap spin column. It will not flow through. Do not fill column higher than the narrow "stem"; for larger volumes, bind multiple additions.

9) Spin in bench-top centrifuge for 30 s at 4,000 g or until all solution has passed through. Protein will be bound within the protein S-Trap. Remove flow through.

⑤ **10)** Wash trapped protein by adding 200 µL of **buffer 5** to the spin column and centrifuging through. Repeat three times. Remove flow through each time. Move S-Trap micro column to a clean 1.7 mL sample tube.

⑥ **11)** Add trypsin or other protease at 1:10 – 1:25 wt:wt in 20 µL **buffer 6**, digestion buffer (blue cap). This buffer is supplied without protease. The protein-trapping matrix is highly hydrophilic and will absorb the solution. Ensure there is no bubble atop the protein trap. Flick if needed to dislodge bubbles.

12) Cap the spin column **loosely** and incubate in a clean tube for 1 hr at 47 °C for trypsin. Most preferably use a water bath or thermomixer. **DO NOT SHAKE**. Shaking will impede performance. Some dripping is harmless. **The cap MUST NOT form an air-tight seal.**

⑥ **13)** After incubation, add 40 µL of **buffer 6** to the S-Trap. Centrifuge peptides out at 1,000 g for 30 sec.

14) Add 40 µL of 0.2% aqueous formic acid. Centrifuge at 1,000 g for 30 sec.

15) Elute hydrophobic peptides with 35 µL of 50% acetonitrile, 0.2% formic acid.

16) Dry down peptides and resuspend as desired (buffer A or MALDI matrix).

PROTIFI™
Innovative proteomics solutions.

S-Trap™ micro universal MS sample prep kit: ≤ 100 µg

Included materials:

- S-Trap™ micro spin columns
- Buffers:
  - ① 2X SDS lysis buffer
  - ② Reducing reagent (TCEP)
  - ③ Capping reagent (MMTS)
  - ④ Phosphoric acid
  - ⑤ Binding/wash buffer (**NOTE:** 8.1 mL methanol must first be added to each bottle)
  - ⑥ Digestion buffer (TEAB)

User-supplied materials:

- HPLC grade methanol. **Add 8.1 mL to each S-Trap binding buffer bottle before use.**
- 0.2% formic acid in MilliQ water
- 0.2% formic acid in 50% HPLC grade acetonitrile
- Protein samples: ≤ 100 µg protein per column
- Protease (e.g. trypsin)

Protocol:

I) DISSOLVE PROTEIN/LYSE SAMPLE

① **1)** Dissolve protein or lyse cells in **22 µL of 1X buffer 1** (white capped vial). A maximum of 100 µg protein should be solubilized. For < 100 µg protein, use the micro kit. Buffer 1 is supplied at 2X so that both liquid and solid samples can be processed. To make 1X buffer 1, dilute 2X buffer 1:1 with MilliQ water.

Sample type	How to dissolve or lyse
Precipitated protein	Add 22 µL of 1X buffer 1 to precipitated protein. Sonicate.
Adherent cell cultures	Wash plates 3x with cold PBS (phosphate buffered saline), removing the washes fully each time. Scrape cells – do not trypsinize – resuspend in PBS and transfer to an appropriate sized tube. Wash plate again with PBS to acquire all cells, transfer the wash to the same tube, pellet and remove wash supernatant. Lyse the appropriate number of cells in 44 µL of 1X buffer preferably with sonication.
Nonadherent cell cultures	Pellet cells gently at 250 g for 10 min at RT. Carefully remove supernatant. Resuspend in cold PBS, filling the tube, then re-pellet. Wash may be repeated. Lyse the appropriate number of cells in 22 µL of 1X buffer preferably with sonication.
Tissue	Preferably, first homogenize sample at cryogenic temperatures (liquid nitrogen or dry ice). Add 22 µL of 1X buffer 1 to homogenized tissue. Sonicate. See www.protifi.com/resources.
Immunoprecipitations	Elute directly from beads with 1X buffer by heating at 70 °C for 10 min. Preferably, use the ultra-high recovery protocol at www.protifi.com/resources.
Liquid protein sample	Add 11 µL of 2X buffer 1 to 11 µL of liquid sample. Sonicate, especially if any flocculent particles are present.
Plasma or serum	Combine ~1.25 µL plasma or serum, 11 µL of 2X buffer 1 and 9 µL of water. This assumes the serum or plasma sample is ~80 mg/mL; quantify protein if needed.
Urine	Follow urine protocol at www.protifi.com/resources.

Note 1: A BCA assay can be used to determine protein concentration of protein in buffer 1.

Note 2: If needed, larger initial volumes of buffer 1 buffer can be used if all volumetric ratios below are kept the same. Larger volumes must be subsequently bound (step 8 below) in volumes up to 100 µL until the entire volume has been passed through the protein trap.

2) Optionally heat sample in buffer 1 at 75 °C for 10 min. Note that aspartic acid – proline (DP) peptide bonds are cleaved by excessive heat.

3) If DNA is present, sheer thoroughly by probe sonication or addition of a nuclease (Benzonase,etc). Dissolve samples and disrupt DNA by sonication or use Benzonase.

Note 3: Benzonase is active sufficiently long in buffer 1 (5% SDS) to degrade DNA when magnesium is present in the lysis buffer. To use benzonase, add $MgCl_2$ to the lysate to 2 mM final concentration. Then add 65 U benzonase per 22 µL of 1X buffer 1.

4) Clarify sample as needed by centrifugation (e.g. 8 min at 13,000 g) or spin filter.

II) REDUCE AND ALKYLATE

5) Add 1 µL of buffer 2 (red cap, 115 mM TCEP) to reduce proteins. Incubate 10 min at 55 °C.

6) Add 2 µL of buffer 3 (yellow cap, 187.5 mM MMTS). Incubate at room temperature for 10 min.

III) BIND AND CLEAN PROTEIN

7) Add 2.5 µL of buffer 4 to the reduced alkylated protein (green cap, 12% aqueous phosphoric acid). Mix.

8) Add 165 µL of buffer 5 (clear cap large bottle, S-Trap binding buffer). Mix. Colloidal protein particulate is instantly formed in this step. Given sufficient protein, the solution may appear translucent. **Be sure to add 8.1 mL of HPLC grade methanol to the bottle before use. Also, ensure complete transfer of the mix including any colloidal material.**

9) With the S-Trap micro in a 1.7 mL tube, add the protein with binding buffer 5 into the spin column. No column preequilibration is necessary.

Note 4: If needed, a larger volume of buffer 1 (SDS lysis/solubilization) may be used. In this case, keep all volumetric ratios the same and load the column multiple times until the full volume has been bound. For example, to load 200 µL of protein plus buffer 5 (S-Trap binding buffer), load 100 µL, centrifuge through, then repeat with the next 100 µL. Proceed to step 10. Do not fill column higher than the narrow "stem."

Note 5: If larger volumes of buffer 1 are used, they may be concentrated ~3-4X by SpeedVac: the S-Trap can handle ≤ 20% SDS. Such concentrations avoid the use of larger volumes of other buffers and multiple loading steps. Most proteins are stable in buffer 1 and proteases are inactive after reduction and alkylation.

10) Centrifuge the micro spin column at 4,000 g for 30 s or until all solution has passed through the S-Trap column. Protein will be bound and trapped within the protein-trapping matrix of the spin column. A vacuum manifold or positive pressure may also be used as desired.

Note 6: After each centrifugation step, make sure that all added solution has gone through the S-Trap column. Centrifuge longer as needed. Unsheered DNA, highly viscous proteins (e.g. from mucosal membranes) or spin column overloading may necessitate significantly longer spin times. Do not exceed 8,000 g with the S-Trap micro spin columns.

11) Wash captured protein by adding 200 µL buffer 5; repeat centrifugation. Repeat this wash step a total of three times. For best results, rotate the S-Trap micro units 180 degrees (like a screw or knob) each

wash. This step is especially impactful when using a fixed-angle rotor and is the equivalent to centrifuging sample tubes first “tab out” then “tab in.” Additional wash(es) may be performed if desired and are recommended if working from unusually high concentrations of detergent, salt, glycerol, etc. Remove wash flow through as necessary. A vacuum manifold or positive pressure may also be used.

IV) DIGEST

12) Move S-Trap micro spin column to the clean 1.7 mL sample tube. The use of a fresh tube prevents contamination of the resultant digestion.

13) Add 20 µL of digestion buffer 6 containing protease at 1:10 – 1:25 wt:wt into the top of the spin column. For effective digestions, do not apply less than 0.75 µg of trypsin. The protein trap is hydrophilic and will absorb the solution. Ensure there is no air bubble between the protease digestion solution and the protein trap. Flick as needed.

14) Cap of the micro column loosely to limit evaporative loss. The cap must not be sealed.

15) Incubate for ≥ 1 hr at 47 °C for trypsin. **DO NOT SHAKE**; shaking will greatly impede performance. Some dripping may occur which does not affect performance. Note that both longer and shorter incubation times are possible and should be optimized or your sample. Overnight incubation at 37 °C in a humid environment is completely acceptable.

Note 7: As with all digestions, optimization of amount of protease, digestion time, buffer and temperature is sample- and enzyme-dependent. Optimize as necessary. Mass spec compatible detergents such as Rapigest™ and ProteaseMax™ are compatible with S-Trap sample digestion and for some sample types have been observed to aid in digestion and sample recovery.

16) Add an additional 40 µL of digestion buffer 6 to the S-Trap spin column containing the digested protein.

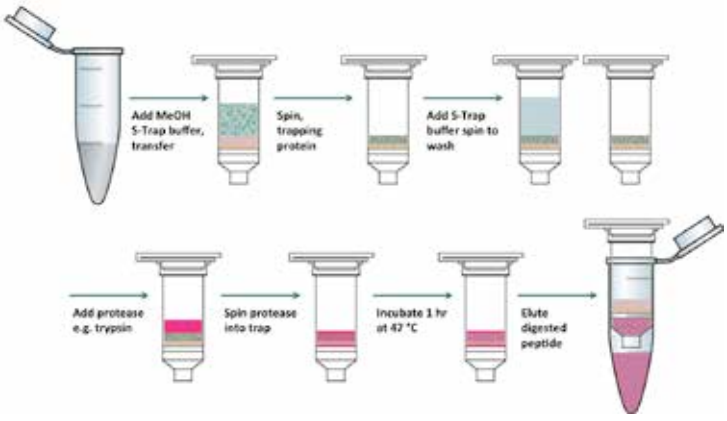
17) Centrifuge at 1,000 g for 60 sec to elute the peptides. This aqueous elution contains the majority of peptides.

18) Add 40 µL of 0.2% aqueous formic acid to the S-Trap protein-trapping matrix and spin through at 1,000 g for 60 sec.

19) Elute hydrophobic peptides with 35 µL of 50% aqueous ACN containing 0.2% formic acid. Other organics may also be used as desired. Centrifuge at 4,000 g for 60 sec for the final elution.

20) Pooled elutions, dry down peptides and resuspend as necessary (e.g. aqueous buffer A for reverse phase chromatography).

Figure 1. Overview of S-Trap protocol.



Troubleshooting

In general, troubleshoot protein capture and digestion by gel analysis of the flow through, washes and elutions. An additional SDS strip (for gel analysis) may be used to determine absolute recovery.

Problem	Possible cause and solutions
Protein not captured or is "missing"	**1.** Applied protein sample was not acidified with buffer 4. This step is necessary for formation of microscopic protein particle captured by the S-Trap. **2.** Colloidal protein particulate must be transferred into the S-Trap unit. If the protein/S-Trap binding buffer solution was centrifuged before addition into the S-Trap, it is possible the protein was pelleted and did not enter the unit. Ensure complete transfer of lysate/binding buffer. **3.** The resultant peptides from some very hydrophobic proteins are poorly soluble in mostly aqueous buffers. Determine theoretical hydrophobicity of expected peptides; HILIC or ERLIC separation may be warranted.
Incomplete protein digestion	**1.** Protease concentration may need to be optimized depending on sample and protease. Try different weight:weight concentrations of protease; try different digestion temperatures; include a mass spec compatible detergent like Rapigest™ or ProteaseMAX™; and/or increase digestion time. Due to evaporation, digestion volume will likely need to be increased with digestion times longer than 1 hr. **2.** Heating of the S-Trap may be insufficient. Ensure entire spin column is exposed to heat and do not have the spin column e.g. sticking out of the top of a heat block where it will receive insufficient heating. **3.** The substrate protein to digest must be exposed to the protease, which requires all pores be filled with the protease solution. Ensure that the protease solution is gently centrifuged into the pores of the protein-trapping matrix. **4.** If the top cap was not applied or not sealed, evaporation may have caused the digestion solution to evaporate before the protease had time to act on the protein. Make sure top cap is applied. **5.** See 2 in "protein not captured or is 'missing'": especially if using a fixed-angle centrifuge, protein may have been pelleted outside the protein-trapping matrix, for example on the side of the S-Trap unit, and thus not exposed to protease. Rotate S-Trap unit 180 degrees as specified in step 11; alternatively use a swing-bucket rotor or a vacuum manifold.
Poor peptide recovery	**1.** The size of S-Trap unit should be matched to the amount of protein to digest. MS-compatible detergents have been found to aid in recovery of low protein levels. Additional elutions may also aid in recovery. **2.** If the digest has dried on the column (for example, if the cap of the micro spin column was not closed), the protein-trapping matrix will need to be rehydrated to solubilize the peptides. Add digestion buffer without enzyme and let sit for 30 min, then centrifuge out. Repeat with the elutions of steps 18 and 19. Additional elutions may assist in peptide recovery. **3.** The protein-trapping matrix retains proteins but not peptides. If digestion is incomplete (see above), poor peptide recovery will result.