S-Trap™ Micro Spin Column Digestion Protocol

1) Lyse cells in 25 µL SDS solublization buffer (5% SDS, 50 mM TEAB pH 7.55). The patent-pending S-Trap™ Micro can handle ≤ 50 – 100 µg of protein.
2) If DNA is present, sheer it thoroughly by probe sonication.
3) Clarify sample as needed by centrifugation for 8 min at 13,000 g.
4) Reduce and alkylate disulfides.
5) Add 2.5 µL 12% phosphoric acid to the lysate solubilized in 25 µL SDS buffer.
6) Add 165 µL of S-Trap binding buffer (90% MeOH, 100 mM final TEAB, pH 7.1) to the acidified lysate
7) Add the acidified lysate/S-Trap buffer mix into the spin column. It will not flow through. Do not fill column higher than the narrow "stem"; for larger volumes, bind multiple additions of ≤ 165 µL.
8) Spin in bench-top centrifuge in a standard 1.7 mL sample tube at 4,000 g until all solution has passed through. Remove flow through.
9) Wash centrifuging through 150 µL S-Trap binding buffer. Remove flow through. Repeat wash three times.
10) Add trypsin at 1:10 – 1:25 wt:wt in 25 µL of 50 mM TEAB, pH 8 to the top of the protein trap. The protein trapping matrix is highly hydrophilic and will absorb the solution. However, ensure there is no bubble atop the protein trap.
11) 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. The cap MUST NOT form an air-tight seal.**
12) Elute peptides with 40 µL each of 50 mM TEAB and then 0.2% aqueous formic acid. Add the first TEAB elution to the trypsin solution prior to any centrifugation. Centrifuge elutions through at 4,000 g.
13) Elute hydrophobic peptides with 35 µL 50% acetonitrile, 0.2% formic acid.
14) Dry down peptides and resuspend as desired (buffer A or MALDI matrix).

PROTIFI™
Innovative proteomics solutions.

S-Trap™ Micro spin column digestion protocol

User-supplied materials:

- 1.7 mL sample tubes
- Protein samples, cells to lyse, serum or immunoprecipitation; up to 50 µg protein per column or less
- Reagents for reduction and alkylation e.g. dithiothreitol (DTT) and iodoacetamide
- Protease (e.g. trypsin)
- Digestion buffer (e.g. 50 mM triethylammonium bicarbonate or ammonium bicarbonate)
- 2x SDS protein solubilization buffer ("lysis buffer": 10% SDS, 100 mM triethylammonium bicarbonate, TEAB, pH 7.55)
- S-Trap protein binding buffer (90% aqueous methanol containing a final concentration of 100 mM TEAB, pH 7.1).
- 12% phosphoric acid

Protocol:

1) Lyse cells or resuspend sample in 25 µL 1x SDS lysis buffer. If sample is liquid, add 12.5 µL 2x lysis buffer to 12.5 µL sample. Final volume can be larger if needed; adjust the volume of S-Trap binding buffer accordingly (step 6 below).[1,2,3,4,5]

2) If DNA is present, sheer it thoroughly by probe sonication. This is essential as DNA will clog the protein trap.

3) Clarify sample as needed by centrifugation (e.g. 8 min at 13,000 g).

4) Reduce and alkylate disulfides by standard techniques (see box insert below).

5) To the SDS lysate, add ~12% aqueous phosphoric acid at 1:10 for a final concentration of ~1.2% phosphoric acid (e.g. 2.5 µL into 25 µL). Mix. This step is essential as the protein trap binds at this pH.

6) Add 165 µL of S-Trap buffer to the 27.5 µL of acidified lysis buffer from step 5 giving a total volume of 192.5 µL.[6] Mix. Colloidal protein particulate is instantly formed in this step. Given sufficient protein, the solution will appear translucent.

7) With the S-Trap micro in a 1.7 mL tube for flow through, add the acidified SDS lysate/MeOH S-Trap buffer mixture into the micro column. No column preequilibration is necessary. Do not add more solution than will fit in the narrow "stem" of the spin column. If initial the SDS lysate volume was higher, load the column multiple times until the full volume has been bound. (E.g. for and initial SDS lysate volume of 50 µL, pass 192.5 µL through twice.)

8) Centrifuge the micro column at 4,000 g until all SDS lysate/S-Trap buffer has passed through the S-Trap column. Protein will be trapped within the protein-trapping matrix of the spin column.[7] Vacuum from below or pressure from above can also be used as desired.

9) Wash captured tprotein by adding 150 µL S-Trap buffer; repeat centrifugation.[7] Repeat wash three times. For best results, rotate the S-Trap micro units like a screw 180 degrees between the centrifugations of steps 8 and 9. (This is especially impactful when using a fixed-angle rotor and is the equivalent to centrifuging sample tubes first "tab out" then "tab in"; a simple mark on the outside edge during the centrifugation of step 8 allows easy rotation of 180 degrees.) Additional wash(es) may be performed if desired and should be performed if contamination is observed by mass spectrometry. Remove wash flow through as necessary. As above vacuum or pressure may also be used.

10) Move S-Trap micro column to a clean 1.7 mL sample tube for the digestion.

11) Add 20 µL of digestion buffer containing protease at 1:10 – 1:25 wt:wt into the top of the micro column.[8] 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.

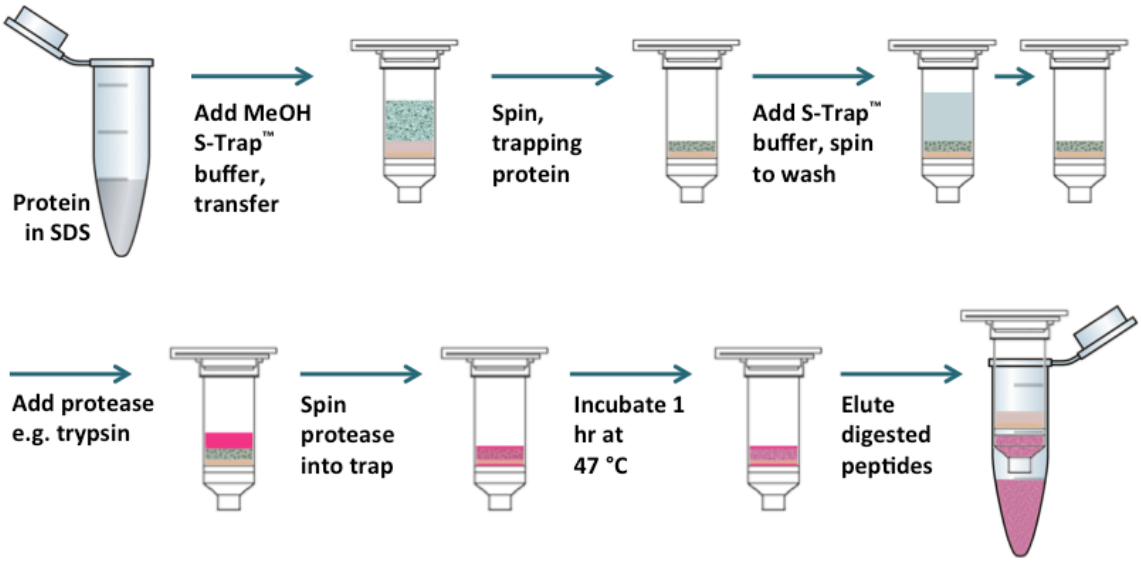
12) Cap the S-Trap tip column to limit evaporative loss.

13) Incubate for 1 hr at 47°C for trypsin.[8] Preferably use a water bath or unmoving thermomixer. Ensure the column is exposed to heat. Do not shake.

14) Elute peptides with 40 µL each of 50 mM TEAB and then 0.2% aqueous formic acid. Add the first TEAB elution to the trypsin solution prior to any centrifugation. Centrifuge elutions through at 4,000 g. Recover hydrophobic peptides with an elution of 35 µL 50% acetonitrile containing 0.2% formic acid. Pool elutions.

15) Dry down eluted peptides and resuspend as desired (e.g. aqueous buffer A for reverse phase chromatography or MALDI matrix)

Figure 1. Overview of S-Trap micro protocol. (Physical dimensions of spin column may differ from the illustration.)



Notes

Note 1: If processing immunoprecipitations (IPs), elute directly with 1x SDS lysis buffer (5% SDS).

Note 2: If processing serum or plasma, mix diluted serum or plasma 1:1 with 2x lysis buffer for a final concentration of 5% SDS.

Note 3: If lysing cells on plate, make sure to wash plates 3x with PBS before lysis, removing PBS washes fully each time.

Note 4: The S-Trap micro is designed for digestion of up to 50 µg protein. S-Trap sample processing are relatively insensitive to SDS concentration such that a three-fold concentration, which would result in a lysate containing approximately 15% SDS, does not affect S-Trap performance. If necessary, concentrate samples e.g. on a SpeedVac.

Note 5: For very low protein loads < 1 µg consider using an MS compatible detergent during digestion to maximize low-level recovery.

Note 6: Note that volumes of S-Trap buffer from 6 – 9x of the acidified SDS protein solution are acceptable. By example if using 30 µL of SDS lysate, use 215 µL. If volumes are larger than the capacity of the spin column, spin through the acidified SDS lysate diluted into S-Trap binding buffer as many times as is required.

Note 7: 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 5,000 g with the S-Trap mini spin columns.

Note 8: 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.

Note 9: 50 mM TEAB or ammonium bicarbonate are recommended for trypsin.

Example reduction and alkylation protocol:

a) Reduce disulfides by adding dithiothreitol (DTT) to the protein solution in SDS to a final concentration of 20 mM. Heat for 10 min at 95 °C.

b) Cool the protein solution to room temperature.

c) Alkylate cysteines by addition of iodoacetamide to a final concentration of 40 mM.

d) Incubate in the dark for 30 min.

e) Remove undissolved matter by centrifugation for 8 min at 13,000 x g.

Note: Samples should not be stored for long periods of time with unreacted iodoacetamde. If samples must be stored after alkylation, inactivate iodoacetamide by the addition of a stoichiometric excess of a sulfhydryl (e.g. cysteine) or by sample precipitation.

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 did not contain SDS. SDS is necessary for the formation of colloidal protein particulate of the size necessary for efficient capture in the protein-trapping matrix. Make sure applied sample contains 5% SDS. **2.** Applied protein sample was not acidified with phosphoric acid. This step is also necessary for formation of colloidal protein particulate. Make sure the SDS solubilized lysate is acidified to 1% final phosphoric acid and is highly acidic. **3.** Colloidal protein particulate must be transferred into the S-Trap unit. If the acidified SDS lysate/MeOH S-Trap buffer solution was centrifuged before addition into the unit, it is possible the protein was pelleted out and did not enter the unit. Ensure complete transfer of acidified lysate/S-Trap buffer.
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. Do not apply less than 0.75 µg of trypsin per trap for efficient digestion. **2.** Heating of the S-Trap may be insufficient. 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 heating. **3.** The substrate protein to digest must be exposed to the protease, which requires all pores be filled with the protease solution; this is the reason for step 12 in the above protocol. Ensure that the protease solution has been force into the pores of the protein-trapping matrix with air pressure and is present above and below the protein-trapping matrix. **4.** See 3 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 column, and not exposed to protease. Rotate S-Trap column 180 degrees as specified in step 9; 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. S-Trap micros are not recommended for > 50 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. **2.** If the digest has dried on the column (for example, if the cap was not applied during incubation), 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 the wash and elution of steps 15 and 16. 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.