S-Trap™ Midi Spin Column Digestion Protocol

1) Lyse cells or resuspend protein in 500 µL SDS solublization buffer (5% SDS, 50 mM TEAB pH 7.55); shear DNA if needed by probe sonication, clarify by centrifugation and reduce and alkylate disulfides. The patent-pending S-Trap™ Midi can process at least 5 mg of protein.
2) Add 50 µL 12% phosphoric acid to the lysate solubilized in 500 µL SDS buffer.
3) Add 3.3 mL of S-Trap buffer (90% MeOH, 100 mM final TEAB, pH 7.1) to the acidified lysate.
4) Add the acidified lysate/S-Trap buffer mix into the spin column.
5) Centrifuge for 1 min at 4,000 g. Remove flow through from tube.
6) Wash by adding 3 mL S-Trap buffer to the spin column and centrifuging. Repeat three times.
7) Discard washes and move column to a clean 15 mL tube.
8) Add trypsin at 1:10 – 1:25 wt:wt in 350 µL of 50 mM TEAB, pH 8. If desired, spin solution into column extremely briefly (pulse only). Return any solution that passes through to the top.
9) Apply 15 mL tube cap **so that it does not make an air-right seal.**
11) Incubate for 1 hr at 47 °C. Most preferably use a water bath or heat block. **DO NOT SHAKE.**
12) Elute peptides sequentially with 500 µL each of 50 mM TEAB (place into column containing digestion buffer and protease before centrifuging out), 0.2% formic acid and 0.2% formic acid in 50% acetonitrile, spinning for 30 s after each.
13) Dry down peptides and resuspend as desired (e.g. for PTM enrichment).

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S-Trap™ midi digestion protocol

Included materials:

- S-Trap™ midi spin columns
- Flow through tubes

User-supplied materials:

- 15 mL sample tubes
- Protein samples, cells to lyse or serum or immunoprecipitation; 10 mg 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 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 500 µL 1x lysis buffer. If sample is liquid, add 250 µL 2x lysis buffer to 250 µL sample. Final volume should not exceed 500 µL.[1,2,3,4,5]

2) If DNA is present, sheer it thoroughly by probe sonication.

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. 50 µL into 500 µL). Mix.

6) Add 3.3 mL of S-Trap buffer to the acidified lysis buffer.[6] Mix. Colloidal protein particulate is instantly formed in this step. Given sufficient protein, the solution will appear translucent.

7) With the S-Trap midi in the flow through tube, add the acidified SDS lysate into the spin column. No column preequilibration is necessary.

8) Centrifuge the midi spin column at 4,000 g for 30 s. Protein will be trapped within the protein-trapping matrix of the spin column.[7] A vacuum manifold may also be used.

9) Wash captured protein by adding 3 mL S-Trap buffer; repeat centrifugation.[7] Repeat wash a total of three times. For best results especially in a fixed angle centrifuge, rotate the S-Trap mini units 180 degrees (like a screw or knob) 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. Remove wash flow through as necessary. A vacuum manifold may also be used.

10) Move S-Trap midi spin column to a clean 15 mL sample tube.

11) Add 350 µL of digestion buffer containing protease at 1:10 – 1:25 wt:wt into the top of the spin column.[8] If desired, spin digestion solution into column very briefly and return any solution that that passes through the spin column to the top of the column.

12) Apply the 15 mL Falcon tube cap **and do not screw it on tightly. It MUST be possible for air pressure to equalize as the S-Trap column increases in temperature or the digest will be forced out by increased pressure.**

13) Incubate for 1 hr at 47°C for trypsin.[8] **DO NOT SHAKE.**

14) Add 500 µL of digestion buffer[9] to the S-Trap spin column containing the protease solution after digestion and before any centrifugation.

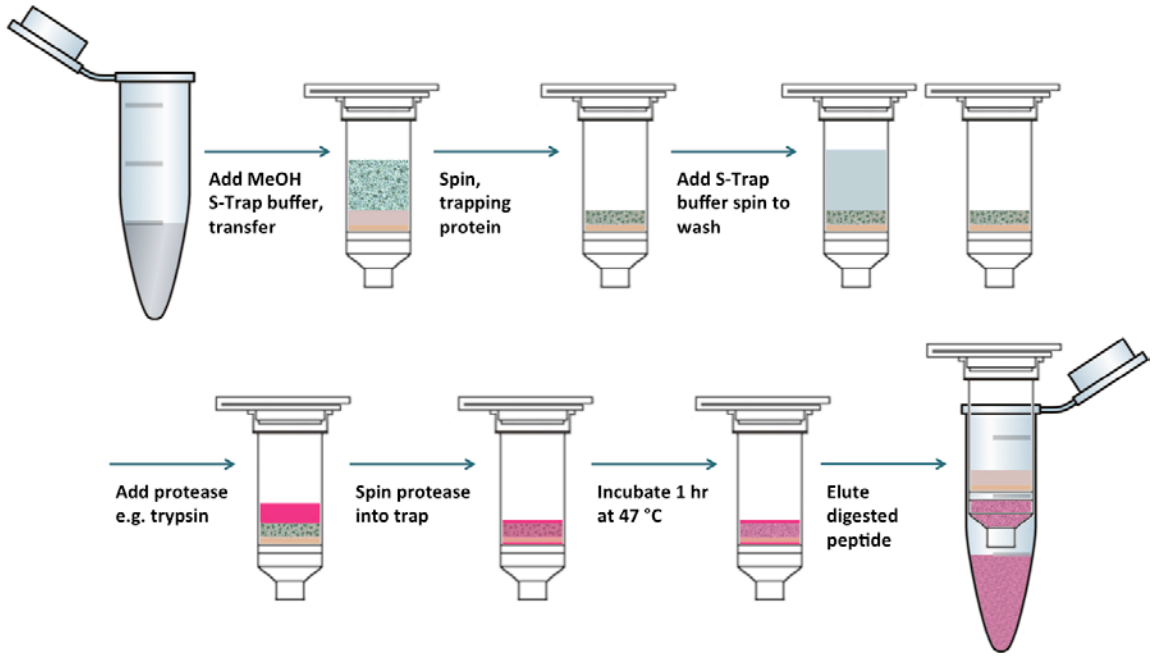
15) Carefully remove the end cap, ensuring not to lose sample, and centrifuge the aqueous elution at 4,000 g for 60 sec. This elution contains the majority of peptides.

16) Add 500 µL of 0.2% aqueous formic acid to the S-Trap protein-trapping matrix and spin through at 4,000 g for 60 sec.

17) Elute peptides with 500 µL of 50% aqueous ACN containing 0.2% formic acid. This elution assists in recovery of hydrophobic peptides.

18) Pooled elutions, dry down peptides and resuspend as necessary (e.g. for PTM enrichment).

Figure 1. Overview of S-Trap protocol.



Notes

Note 1: If processing large-scale 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 midi is designed for digestion of up to 10 mg protein in volume of 500 µL of reduced, alkylated and acidified lysate. 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: The S-Trap midi is not recommended for protein loads < 100 µg.

Note 6: Note that volumes of S-Trap buffer from 6 – 9x of the acidified SDS protein solution are acceptable.

Note 7: After each centrifugation step, make sure that all added solution has gone through the S-Trap tip. 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 midi 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 100 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 sufficient 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 protein sample is acidified to a final concentration of 1.2% 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. **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; this is the reason for steps 11 – 13 in the above protocol. Ensure that the protease solution is gently centrifuged into the pores of the protein-trapping matrix. **4.** With very large quantities of protein (e.g. 10 mg), the precipitated protein may be visible on top of the protein-trapping matrix. The physical volume of colloidal protein precipitant may necessitate additional digestion buffer (~ 400 – 500 µL rather than 350 µL). **5.** If the end cap was not applied or was not sealed, the protease digestion solution may have flowed out of the S-Trap protein-trapping matrix before the protease digested the protein. Ensure that the column is sealed prior to incubation. **6.** 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. **7.** 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 unit, and thus not exposed to protease. Rotate S-Trap unit 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 midis are not recommended for < 100 µg; use S-Trap minis or micros. 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 midi 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 17 and 18. 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.