S-Trap™ Mini Spin Column Digestion Protocol Quick Card

1) Lyse cells, extract or resuspend protein in 50 µL SDS solublization buffer (5% SDS, 50 mM TEAB, pH 7.55). As needed, shear DNA by sonication or addition of Benzonase. Clarify by centrifugation. Reduce and alkylate disulfides in SDS lysis buffer. The patent-pending S-Trap™ Minis optimally digest 100 – 300 µg of protein.
2) Add 5 µL 12% phosphoric acid to the 50 µL of SDS solubilization buffer.
3) Add 350 µL of S-Trap binding buffer (90% MeOH, 100 mM TEAB final; pH 7.1) to the acidified lysate.
4) Add the acidified SDS lysate/S-Trap buffer mix into the spin column.
5) 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 trap. Remove flow through.
6) Wash trapped protein by adding 400 µL S-Trap binding buffer to the spin column and centrifuging through. Repeat three times. Remove flow through. Perform the washes in one 2 mL tube, then transfer the spin column to one of the included fresh 2 mL tubes. This prevents contamination of the digestion.
7) Add trypsin at 1:10 – 1:25 wt:wt in 125 µL of 50 mM TEAB, pH 8. Spin protease into column briefly; return any solution that passes through to the top of the column. The protein-trapping matrix is highly hydrophilic and will absorb the solution. However, ensure there is no bubble atop the protein trap. Flick if needed.
8) Cap the spin column and incubate in a clean tube for ≥ 1 hr at 47 °C (for trypsin). Most preferably use a water bath. **DO NOT SHAKE.** Shaking will impede performance. Some dripping is harmless.
9) Elute peptides with 80 µL each of 50 mM TEAB and then 0.2% aqueous formic acid. Add the TEAB elution to the trap with protease prior to centrifugation. Centrifuge elutions at 1,000 g.
10) Elute hydrophobic peptides with 80 µL of 50% acetonitrile, 0.2% formic acid.
11) Dry down peptides and resuspend as desired (buffer A or MALDI matrix).

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Innovative proteomics solutions.

S-Trap™ mini (100 µg – 300 µg) digestion protocol

Included materials:

- S-Trap™ mini spin columns
- 2 mL flow through tubes

User-supplied materials:

- 1.7 mL sample tubes
- Protein samples, cells to lyse, serum or immunoprecipitation; 300 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 tris)
- 2x SDS protein solubilization buffer ("lysis buffer": 10% SDS, 100 mM triethylammonium bicarbonate, TEAB, pH 7.55; pH should be adjusted with phosphoric acid)
- S-Trap binding buffer (90% aqueous methanol containing a final concentration of 100 mM TEAB, pH 7.1; make by adding methanol to pH-adjusted 1 M TEAB until the final volume is 10x the initial volume of 1 M TEAB).
- 12% phosphoric acid

Protocol:

1) Lyse cells or resuspend sample in 50 µL 1x lysis buffer. If sample is liquid, add 25 µL 2x lysis buffer to 25 µL sample. The initial volume of SDS lysis buffer can be larger if needed. In the case of larger volumes, ensure that the ratio of SDS lysis buffer to S-Trap binding buffer remains at 1:7 (step 6 below). To subsequently bind larger volumes (step 7 below), sequentially centrifuge through volumes of up to 600 µL of acidified SDS lysate diluted with S-Trap binding buffer until the entire volume has been passed through the protein trap.[1,2,3,4,5]

2) If DNA is present, sheer it thoroughly by probe sonication or addition of a nuclease such as Benzonase.

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. 5 µL into 50 µL). Mix.

6) Add 350 µL of S-Trap binding buffer to the acidified lysis buffer.[6] (For starting volumes > 50 µL of SDS lysis buffer, maintain a 1:7 v:v ratio of SDS lysis buffer to S-Trap binding buffer.) Mix. Colloidal protein particulate is instantly formed in this step. Given sufficient protein, the solution may appear translucent.

7) With the S-Trap mini in a 2 mL receiver tube, add the acidified SDS lysate/S-Trap binding buffer mix into the spin column. No column preequilibration is necessary. Do not add more solution than will fit in the lower, straight portion spin column. If initial the SDS lysate volume was higher, load the column multiple times until the full volume has been bound. For example, to load 900 µL of acidified SDS lysate diluted with S-Trap binding buffer, load 450 µL, centrifuge through, then repeat with the next 450 µL and proceed to step 8.

8) Centrifuge the mini spin column at 4,000 g for 30 s or until all SDS lysate/S-Trap buffer has passed through the S-Trap column. Protein will be bound and trapped within the protein-trapping matrix of the spin column.[7] A vacuum manifold or positive pressure may also be used as desired.

9) Wash captured protein by adding 400 µL S-Trap buffer; repeat centrifugation.[7] Repeat this wash step a total of three times, then transfer the S-Trap to a fresh 2 mL tube; the use of a fresh tube prevents contamination of the resultant digestion. For best results, rotate the S-Trap mini units 180 degrees (like a screw or knob) between the centrifugations of steps 8 and 9. (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.

10) Move S-Trap mini spin column to the clean 2 mL sample tube.

11) Add 125 µL of digestion buffer containing protease at 1:10 – 1:25 wt:wt into the top of the spin column.[8] Spin the protease solution into the column briefly and return any solution that passes through to the top of the column. The protein trap is highly hydrophilic and will absorb the digestion buffer.

12) Close the cap of the mini spin column to limit evaporative loss. The column lid is designed to prevent pressure build up, which would otherwise force the digestion from the protein trap, while limiting evaporative loss.

13) Incubate for ≥ 1 hr at 47 °C for trypsin.[8] **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.

14) Add 80 µL of digestion buffer[9] to the S-Trap spin column containing the protease.

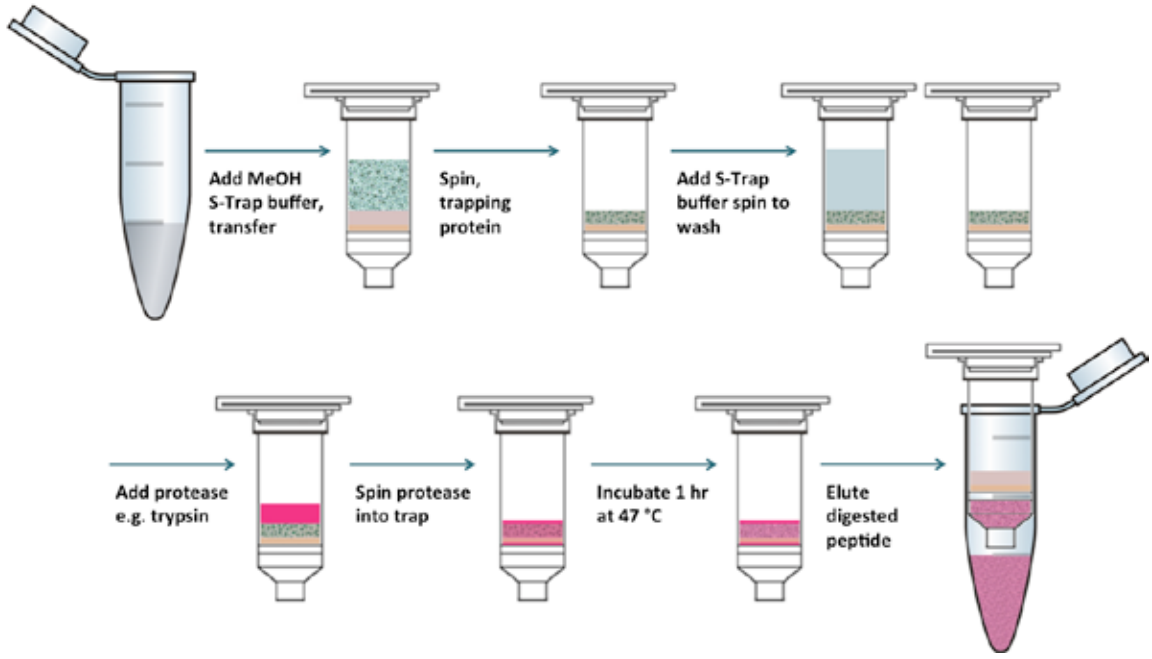
15) Centrifuge at 1,000 g for 60 sec to elute the peptides. This aqueous elution contains the majority of peptides.

16) Add 80 µL of 0.2% aqueous formic acid to the S-Trap protein-trapping matrix and spin through at 1,000 g for 60 sec.

17) Elute peptides with 80 µL of 50% aqueous ACN containing 0.2% formic acid. This elution assists in recovery of hydrophobic peptides. Other organics may also be used as desired. Centrifuge at 4,000 g for 60 sec for the final elution.

18) 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.



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 cold PBS (phosphate buffered saline) before lysis, removing PBS washes fully each time.

Note 4: The S-Trap mini is designed for digestion of up to 300 µg protein in volume of 50 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. It is possible to use larger volumes but the acidified lysate diluted into S-Trap binding buffer must be applied in multiple aliquots.

Note 5: The S-Trap mini is not recommended for protein loads < 50 – 100 µg. It will function for lower loads but will have reduced recovery and digestion kinetics.

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 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 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 a minimum of 2% 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. **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.** If the end plug 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. **5.** 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. **6.** 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 minis are not recommended for < 10 µg; use S-Trap 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 mini 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.