S-Trap™ 96-well plate digestion protocol

Included materials:

- S-Trap™ 96-well digestion plate
- S-Trap™ 96-well digestion plate cover

User-supplied materials:

- Receiver plates (two recommended: one for flow through and washes and one for elutions)
- Protein samples, cells to lyse, serum or immunoprecipitation; 100 - 250 µg protein per well
- Reagents for reduction and alkylation e.g. dithiothreitol (DTT) and iodoacetamide
- Protease (e.g. trypsin)
- Digestion buffer (preferably volatile buffers 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 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 50 µL 1x lysis buffer. If sample is liquid, add 25 µL 2x lysis buffer to 25 µL sample. Final volume should not exceed 50 µL.[1,2,3,4,5]

2) If DNA is present, sheer it thoroughly by probe sonication. **This step is essential as unsheered DNA will clog the protein trap.** A nuclease such as Benzonase™ may also be used.

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] Mix. Colloidal protein particulate is instantly formed in this step. Given sufficient protein, the solution will appear translucent.

7) With the S-Trap plate atop the flow through 96 well receiver, add the acidified SDS lysate into the spin column. No column preequilibration is necessary. Solution typically beings to drip through immediately.

8) Centrifuge the plate at 1,500 g for 2 min or until all solution has passed through. Protein will be trapped within the protein-trapping matrix of the plate.[7] A vacuum manifold may also be used provided that all wells have flow behavior; this may not be the case for all samples.

9) Wash captured protein with three washes of 200 µL of S-Trap binding buffer, followed by centrifugation at 1,500 g for 2 min.[7] Additional wash(es) may be performed if desired. Washes may be captured with the flow through. A vacuum manifold may also be used if the wells have similar flow behavior.

10) Move S-Trap digestion plate on top of a clean receiver plate.

11) Add 125 µL of digestion buffer containing protease at 1:10 – 1:25 wt:wt into the top of the wells.[8] Ensure the protein capture matrix is fully covered by the digestion solution.

12) Loosely cover the digestion plate to limit evaporative loss. **It must not make an air-tight seal.**

13) Incubate for 1 hr at 47°C for trypsin.[8] Some dripping may occur during incubation; this is not of concern. DO NOT SHAKE.

14) Add 80 µL of digestion buffer[9] to all wells of the S-Trap digestion plate. Centrifuge the plate at 1,500 g for 2 min or until all solution has passed through. Do not centrifuge the plate prior to addition of 80 µL of digestion buffer used in this first elution.

15) Add 80 µL of 0.2% aqueous formic acid to all wells of the S-Trap digestion plate and spin through at 1,500 g for 2 min.

18) Further elute peptides with 80 µL of 50% aqueous ACN containing 0.2% formic acid. This elution assists in recovery of hydrophobic peptides. The final ACN concentration will be around 10% v/v.

19) Pool elutions, dry down peptides and resuspend as necessary (e.g. aqueous buffer A for reverse phase chromatography). Note that while S-Trap sample processing reproducibly digests proteins solubilized in SDS, peptides are not necessarily soluble in buffer A of reverse-phase chromatography.

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 96-well digestion plate is designed for digestion of up to 250 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.

Note 5: The S-Trap 96-well digestion plate is not recommended for protein loads < 50 µg.

Note 6: Note that volumes of S-Trap binding 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 plate. Centrifuge longer as needed. Unsheered DNA, highly viscous proteins (e.g. from mucosal membranes) or overloading with protein or contaminants may necessitate significantly longer spin times. Do not exceed 2,000 g with the S-Trap 96-well plate.

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. **2.** Heating of the S-Trap may be insufficient. Ensure entire spin plate is exposed to even heat. **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 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.
Poor peptide recovery	**1.** The size of S-Trap sample processing unit should be matched to the amount of protein to digest. The 96-well S-Trap plate is not recommended for < 50 µg. 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 plate (for example, the plate was forgotton), 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.