S-Trap™ 96-well MS sample prep kit: 100 – 300 µg

Required equipment and materials:

- 1.7 mL and 2 mL sample tubes
- Single- and/or multi-channel pipettors with corresponding tips (P2, P20, P200, P1000)
- Benchtop centrifuge
- Vortex mixer
- Heat block set to 55 °C
- Water bath or humidified incubator set to 47 °C
- SpeedVac or lyophilizer
- Vacuum manifold (optional; Supelco Visiprep SPE Vacuum Manifold is recommended)
- Sonicator, PIXUL or Covaris ultrasonicators; automation. Optional and recommended; see www.protifi.com/pixul and www.protifi.com/A200.

Required reagents and solutions:

- Protein sample (**100 – 300 µg**)
- Protease of choice (e.g. trypsin; 10 µg per 100 µg of sample)
- LC/MS grade methanol (**MeOH**)
- Benzonase and 50 mM $MgCl_2$ (optional)

Provided materials:

- S-Trap 96-well digestion plate
- 96-well 2 ml receiver plate for flow through and washes
- 96-well 1 ml receiver plate for elutions
- Silicone mat for 96-well elution plate

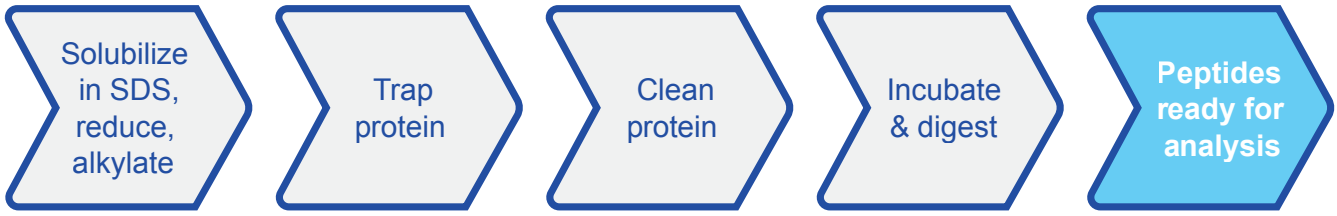
Provided solutions:

Buffer	Solution	Composition	Volume	Storage
①	2X lysis buffer (for liquid samples)	10% SDS, 100 mM TEAB pH 8.5	2 x 1.5 mL	1 year at RT
②	Reductant	120 mM TCEP in water	250 µL	Store at -20 ºC; 1 year
③	Alkylator	500 mM MMTS in isopropanol	250 µL	1 month at 4 ºC
④	Acidifier, 96/micro (27.5% phosphoric acid)	Phosphoric acid diluted to 27.5% with water	550 µL	1 year at RT if kept sealed; can absorb CO_2 from air over time
⑤	Binding/wash buffer	100 mM TEAB (final) in 90% methanol. **27 mL methanol must first be added to each bottle.**	4 x 3 mL in 30 mL bottles	1 month at RT; 1 year at 4 ºC
⑥	Digestion buffer	50 mM TEAB	28.5 mL	1 month at RT; 1 year at 4 ºC
⑦	Elution buffer	50% acetonitrile, 50 mM TEAB pH 8.5	9.75 mL	1 month at RT; 1 year at 4 ºC

Solutions not included:

Solution	Composition	pH	Storage
Trypsin stock solution	Trypsin resuspended in Digestion buffer (Buffer 6) at a concentration of 1 mg/mL	Unadjusted pH at 8.5	Aliquot and freeze at -80 ºC
Methanol	Added to Binding/wash buffer prior to use	Unadjusted	Follow manufacturer's guidance

S-Trap 96-well plate protocol



Solubilize in SDS, reduce, alkylate

1) For a liquid sample, add 25 µL Buffer 1 (white cap) to 25 µL sample to yield a final volume of 50 µL. For solid samples (pelleted cells, protein pellets, or tissue) dilute Buffer 1, which is provided as a 2X solution, 1:1 with MilliQ water to make 1X lysis buffer and add 50 µL of 1X lysis buffer to the sample.

- For samples of different volumes or types, see Appendix A and Appendix B at end of protocol.
- SDS concentration must be ≥ 2% for good recovery.
- High concentrations of SDS (up to 15%) do not affect S-Trap performance. If necessary, concentrate samples on a SpeedVac or lyophilizer.

2) Sonicate or vortex to lyse sample and dissolve proteins.

3) If the sample is viscous, sheer DNA thoroughly via sonication or treatment with Benzonase. This is essential as DNA can clog the S-Trap column.

Approach	Steps
PIXUL ultrasonication	Ultrasonicate at 20 °C for 5 min using 50 N pulses at 1 kHz with a 20 Hz burst rate.
Covaris ultrasonication	On a S220, ultrasonicate at 175 W using 200 cycles per burst and a 10% duty factor at 20 °C for 6 minutes.
Probe or horn sonicator	Settings depend on the power of the sonicator. Test power setting and length of sonication with a 1.5 mg/mL solution of salmon sperm DNA, sonicating until the viscosity is indistinguishable from an aqueous solution.
Benzonase	Add $MgCl_2$ to lysis buffer to yield a final concentration of 2 mM. Add 100 units Benzonase per 50 µg of protein and incubate at RT for 5 min.

4) As needed, clarify sample of debris by centrifugation (e.g. 8 min at 13,000 g and take the supernatant).

- **Note:** insoluble pellets may contain proteins of interest. If desired, insoluble sample can be processed as solids atop a separate S-Trap. Perform all following steps on the solid sample, and homogenize the insoluble sample as much as possible before sample processing.

5) Reduce: add 2 µL of Buffer 2 (red cap) and incubate at 55 °C for 15 min.

6) Alkylate disulfides: add 2 µL of Buffer 3 (yellow cap) and incubate at Room Temperature for 10 min.

7) Add 5 µL of Buffer 4 (green cap) and vortex.

- This step is essential to completely denature proteins and trap them efficiently.
- The pH will be ≤ 1. If the sample pH is not ≤ 1, add additional acidifier (Buffer 4) to reach pH ≤ 1.
- The acidifier is different between S-Trap micro and mini kits.

Trap protein

8) Add 27 mL of LC/MS grade methanol to Buffer 5 bottles (30 ml bottle with clear cap) before use.

⑤ **9)** Add 350 µL of Buffer 5 (30 ml bottle with clear cap **with methanol added**) to the sample and mix.

- Sufficient protein may make the colloidal protein particulate visible, giving a translucent appearance.
- **All sample** – including any resulting colloid – must be transferred to the S-Trap. Sample agitation by pipetting up and down may be necessary to transfer all sample including colloid. Do not centrifuge at this point.

10) Place S-Trap plate on the provided 2 mL receiver plate for waste flow through.

11) Apply sample to S-Trap plate.

- To load plate manually, aspirate sample(s) into the pipette tip(s), pipetting up and down several times to agitate the binding buffer/sample mix. Then move the pipette tip(s) containing the sample(s) over the intended well(s) of the 96-well S-Trap plate, touch the pipette tip(s) to the side of the wells 1 mm above the S-Trap matrix (without touching the matrix) and dispense the solution.
- All sample, including anything insoluble, must be transferred into the S-Trap.
- No column preequilibration is necessary.
- For larger initial volumes, see Appendix A and load the column multiple times with volumes ≤ 450 µL.

12) Centrifuge the S-Trap plate at 2,000 g in a swing bucket rotor for 2 min to trap proteins.

- Visually confirm all sample has passed through the column; if not, centrifuge again until all solution has passed through.
- Negative pressure via a vacuum manifold or positive pressure from above can also be used.
- Plate may be centrifuged until no solution is visible atop the trapping matrix. See above notes on DNA sheering.

Clean protein

⑤ **13) First two washes:** add 200 µL of Buffer 5; centrifuge at 2,000 g for 2 min in a swing bucket rotor or until all solution has passed through the matrix. Repeat this wash step once more and discard flow through as necessary.

- Additional wash(es) may be performed and should be performed if contamination is observed by mass spectrometry. You cannot over wash mammalian proteins with binding/wash buffer.
- If the sample has particularly hydrophobic contaminants, such as lipids in brain, bone marrow or adipose tissue, or wax in FFPE, bind protein, wash three times with 450 µL 50% $CHCl_3$/50% MeOH, then perform three washes with standard methanolic binding/wash buffer as normal.
- Vacuum or pressure may also be used at this step. For large initial sample volumes, use of a vacuum manifold is highly recommended.
- 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 tissues) or spin column overloading may necessitate significantly longer spin times.

14) **Third and last wash:** add 200 µL of Buffer 5; centrifuge at 2,000 g for 5 min in a swing bucket rotor to fully remove residual Buffer 5. Failure to do so may result in dripping.

15) Transfer S-Trap plate to the provided 1 mL collection plate for the digestion.

Incubate and digest protein

⑥ **16)** Dilute trypsin stock solution (or another enzyme stock solution) with Buffer 6 so the enzyme is in a 1:10 weight to weight ratio to the amount of protein trapped on the column. The final volume of digestion buffer containing enzyme added to each column will be 125 µL. By example, for 200 µg of protein trapped on the S-Trap column, 20 µg of trypsin dissolved in a final volume of 125 µL will be applied.

⑥ **17)** Add 125 µL of protease diluted in Buffer 6 (prepared in step 15), into the S-Trap.

- Do not apply less than 10 µg of trypsin per well for effective digestion.

- 50 mM TEAB is provided as Buffer 6 and recommended for trypsin or trypsin/lys-C mixes. If using a different enzyme, check enzyme specifications.
- The S-Trap is hydrophilic and will absorb the solution. Apply it directly above the trapping matrix and do not damage it with pipette tips.
- Other proteases than trypsin can be used, however they may require different digestion buffers, temperatures, pHes, metal cofactors, and/or times. S-Traps have been found to be compatible with Tryp-N, Lys-N, Lys-C, Arg-C, Glu-C, chymotrypsin, elastase and pepsin. Trypsin/lys-C mixes generally perform better than trypsin alone. S-Traps can also be used for glycomics with PNGase F.
- Mass spec compatible detergents such as Rapigest™ are compatible with S-Trap sample digestion and for some sample types have been observed to aid in digestion. ProteaseMAX™ should not be used at elevated digestion temperatures due to accelerated autolysis. Trypsin/lys-C mixes generally perform better than trypsin alone.

18) Cover the S-Trap plate with the provided lid to limit evaporative loss. **Do not seal the plate** (as with adhesive plate foils) as pressure buildup will force the solution prematurely through the trapping matrix.

19) Incubate for 1 hr at 47 °C for trypsin or trypsin/lys-C, or overnight at 37 °C in a water-saturated atmosphere (see below). After 1 hr add 75 µL more of Buffer 6 and return plate to the incubator for another 1 hr or overnight.

- Ensure the entire plate is exposed to heat. Do not shake.
- After 1 hr of heating, add 75 µL more of Buffer 6. If doing a 47 °C digestion, incubate for 1 hr more at 47 °C. If doing an overnight digestion, return to 37 °C.
- Preload dry incubators with beakers of water to saturate the air with water vapor and limit evaporation; let the beakers come to temperature before using the incubator. If using a thermomixer, fill unused wells with water and cap. If samples appear to be drying out despite a water-saturated atmosphere, add additional digestion buffer to compensate for evaporative loss.
- **As with all digestions, optimization of amount of protease, digestion time, buffer and temperature is sample- and enzyme-dependent.** Peptides all have their own unique digestion kinetics: optimize as necessary. See https://pubs.acs.org/doi/abs/10.1021/pr100656u.

Elute peptides for analysis

20) Remove plate from the incubator and add 75 µL of Buffer 6 (Elution buffer 1 is the same as Buffer 6) to the S-Trap, then centrifuge (2,000 g, 2 min).

- Do not centrifuge the digestion through before applying elution buffer 1. Apply elution buffer 1 directly into the trap containing the digestion buffer that was incubated.

21) Add 80 µL of Buffer 7 (50% acetonitrile in 50 mM TEAB) to the S-Trap then centrifuge (2,000 g, 2 min). This elution assists in recovery of hydrophobic peptides. Other organics may also be used as needed.

22) Pool eluted peptides, dry down and resuspend as desired (e.g. aqueous buffer A such as 5% acetonitrile, 0.1% formic acid for reverse phase chromatography or MALDI matrix).

Appendix A – Buffer additions based on initial sample volumes

- If the protein sample does not contain SDS, either add the same volume of 2X lysis buffer, add dry SDS or add SDS stock to a minimum of 2% SDS.
- The amount of reductant to add is always 1/23 of the initial sample volume. E.g. for 46 µL, add 2 µL.
- The amount of alkylator to add is always 1/23 of the initial sample volume. E.g. for 46 µL, add 2 µL.
- The amount of acidifier to add is always 1/10 of the initial sample volume, plus the added volumes of TCEP and MMTS. E.g. for 50 µL, add 50 µL.
- The amount of binding/wash buffer to add is 6X the total volume of sample (initial volume plus other reagents). E.g. for (50 µL + 5 µL) x 6 = 330 µL, add 330 µL. The amount of binding/wash buffer can be up to 9X; 350 µL in step 8 is has been kept for historical reasons and is not an error.

Sample	Buffer 1 diluted 1:1 with water (µL)	Buffer 1 (µL)	Buffer 2 (µL)	Buffer 3 (µL)	Buffer 4 (µL)	Buffer 5 (µL)
Pelleted cells	46	—	2	2	5	350
Protein pellet	46	—	2	2	5	350
11.5 µL liquid	—	11.5	1	1	2.5	175
15 µL liquid	—	15	1.3	1.3	3.3	228
30 µL liquid	—	30	2.6	2.6	6.5	457
50 µL liquid	—	50	4.3	4.3	10.9	913
100 µL liquid	—	100	8.7	8.7	21.7	1,522

Appendix B – Sample type recommendations

Note that protein concentration should be assayed by BCA for all samples except IPs.

Sample type	Recommendation
Adherent cells on plate	Wash cell plate 3x with cold PBS in rapid succession ensuring complete PBS rinse removal each time. Do not let cells dry out. For best results, tip the plate containing the last PBS wash and use a cell scraper to bring all cells to the bottom corner of the plate. Remove cells, pellet and lyse with 1X lysis buffer using sonication.
Bacteria and archaea	Disrupt the cell walls using beads beating, cryopulverization and/or ultrasonication in 1X lysis buffer. Degree of homogenization needed is strongly a function of the species of microorganism. Monitor by microscopy. See https://doi.org/10.3390/microorganisms8030413.
Bile	Mix bile 1:1 with 2X lysis buffer and proceed with S-Trap digestion. See https://doi.org/10.1016/j.jprot.2020.103984.
Bioreactor supernatant	Mix bioreactor supernatant 1:1 with 2X lysis buffer and proceed with the S-Trap digestion. Polymeric surfactants such as Pluronic F68 will be fully removed. See https://doi.org/10.1021/acs.jproteome.0c00106.
Exosomes	Purify exosomes by differential centrifugation. Dissolve exosomes in 1X lysis buffer preferably with ultrasonication. Proceed with S-Trap digestion. See https://doi.org/10.1038/s41598-018-37002-x.
Formalin-fixed paraffin-embedded (FFPE) samples, scrolls or cores	Follow the HYPERsol protocol (https://doi.org/10.1021/acs.jproteome.9b00686) with the following alteration: allow cores or scrolls to hydrate in 1X lysis buffer overnight. The next day, ultrasonicate for 5 min or until pieces have been dissolved. For PIXUL ultrasonication, use the following settings: 50 N pulse at 1 kHz with a 20 Hz burst rate. Perform additional washes with 50% $CHCl_3$/50% MeOH as described above in “Clean protein.”
IP	Elute directly with 1X SDS lysis buffer (5% SDS) and use the high recovery protocol.
Membrane fractions/proteins	Isolate membrane fraction; ultracentrifugation or Triton X-114 phase separation can be used. Dissolve membrane fraction in 1X lysis buffer with ultrasonication and proceed with S-Trap digestion. See https://doi.org/10.1038/s41594-020-0425-5.
Laser capture microdissection (LCM) pieces	Dissolve LCM pieces in 1X lysis buffer with heating and use the high recovery S-Trap protocol. See https://doi.org/10.1186/s12014-020-09287-6. Note this reference analyzed haematoxylin and eosin (H&E) stained, formalin-fixed paraffin-embedded (FFPE) tissues.
Nonadherent cells	Gently pellet cells and resuspend in the maximal practical volume of cold PBS (e.g. ~10 mL in a 15 mL Falcon tube). Repeat rinse 3x, then pellet and lyse with 1X lysis buffer using ultrasonication.
PBMC fractions, COVID samples	Lyse cells or sample in 1X lysis buffer and proceed with the S-Trap digestion. See https://doi.org/10.1021/ acs.jproteome.0c00365.
Saliva or sputum	Measure protein concentration and aliquot saliva sufficient for 100 µg. Add a 20% stock solution of SDS to 2% SDS. Reduce and alkylate to liquefy, then follow the standard S-Trap protocol. See https://doi.org/10.1021/acs.jproteome.8b00505.
Serum/plasma	Dilute serum/plasma 1:20 with PBS and mix 12.5 µL diluted serum with 12.5 µL 2X lysis buffer. See https://doi.org/10.1016/j.jprot.2020.103645.
Stool	Dissolve stool with 1X lysis buffer using agitation (bead beating or ultrasonication) and proceed with S-Trap digestion. See https://doi.org/10.1128/mSystems.00200-20.
Tissues	**Do not allow tissue to defrost until immediately before lysis.** As needed and depending on size, crush frozen tissue with a precooled pellet pestle, mortar and pestle, hammer, Covaris CryoPrep, etc. In all cases be extremely cautious to not lose pieces of tissue as **THEY WILL FLY OUT**. Ideally, cryopulverize and/or ultrasonication in 1X lysis buffer. Add frozen 1X lysis buffer at 10X – 50X wt:wt with tissue. The degree of homogenization needed is strongly a function of the kind of tissue. Bone will require demineralization with EDTA; larger pieces may require days. Length and power of ultrasonication must be optimized for each tissue.
Urine	Aliquot 0.5 mL urine containing at least 100 µg of protein and add 2 mL of cold acetone. Precipitate the proteins overnight at −20 °C. Centrifuged samples at 20,000 g for 10 min and dissolve the pellet in lysis buffer. See https://doi.org/10.1021/acs. jproteome.9b00772.
Yeast	Disrupt the cell walls using beads beating, cryopulverization, heat and/or ultrasonication in 1X lysis buffer. Monitor cell wall disruption by microscopy.

Appendix C – 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 causes and solutions
Protein not captured or is "missing"	**1.** Applied protein sample did not contain sufficient SDS. SDS is necessary for efficient capture in the protein-trapping matrix. Make sure applied sample contains 5% SDS. **2.** Applied protein sample was not acidified properly with phosphoric acid. This step is also necessary for efficient protein denaturation and capture. Make sure the SDS solubilized protein sample is acidified to a final concentration of 2.5% phosphoric acid and is highly acidic. Check pH is ≤ 1. **3.** All protein, including any particulate or colloid, 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 1 µg of trypsin per trap for efficient digestion; other proteases may require different minimum amounts. Trypin/Lys-C mixes work better than trypsin alone. **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.** Bubbles atop the trap may prevent the sample from being exposed to the digestion protease; ensure no bubbles are present by visual inspection. If any bubbles are present, flick until they float to the top. Protease may be applied directly above the trap with a thin pipette tip. Note that the protein trap is highly hydrophilic; the protease solution will be quickly "sucked" into the trap in the absence of a bubble. With careful observation, this behavior can be seen – it lasts < 1 sec – and is verification that the protease has been absorbed into the trap. **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 the S-Trap column 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. S-Trap micros are not recommended for > 100 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, or the column was forgotten), 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. Additional elutions may assist in peptide recovery. Concentrate by lyophilization. **3.** The protein-trapping matrix retains proteins but not peptides. If digestion is incomplete (see above), poor peptide recovery will result. Digestion can be monitored by gel as well as mass spec analyses.