Protein digestion by S-Trap^{TM} micro MS sample preparation

Adapted from PROTIFI company

Introduction

The following protocol describes a method for generation of tryptic peptides from crude lysates for LC-MS analysis. The method allows analysis of detergent lysed cells and tissues. Therefore, it is particularly suitable for studying entire proteomes and fractions containing biological membranes. We have replaced commercial S-Trap columns by ‘’S-Trap-like’’ columns that perform as efficiently as the commercial one for a fraction of the price. Those inexpensive columns are available from NEB Canada company (Monarch® Spin PCR & DNA Cleanup Kit (cat: T1130L).

1. Materials

Solutions and Reagents (use MS-grade water for all solutions)

-1 M TEAB stock.

-1X lysis buffer: 5% sodium dodecyl sulfate (SDS), 50 mM triethylammonium bicarbonate (TEAB), pH 8.5.

-Reductant: 120 mM Tris(2-carboxyethyl)phosphine (TCEP) in water.

-Alkylator: 500 mM of chloroacetamide (ClAA) in water.

-Acidifier: Phosphoric acid diluted to 27.5% with water.

-Binding/wash buffer: 100 mM TEAB (final) in 90% methanol.

-Trypsin Stock: 1 µg/µl in 50 mM acetic acid.

-Digestion buffer: 200 mM TEAB containing sufficient trypsin stock solution to yield 1 µg trypsin per 10 µg sample (weight:weight) (or 5 ug maximum of trypsin for an IP digestion) in 20 µl of 200 mM TEAB; no less than 1 µg trypsin per micro column.

-Elution buffer 1: 50 mM TEAB in water.

-Elution buffer 2: 0.2% formic acid in water.

-Elution buffer 3: 50% acetonitrile in water.

Equipment

1.5 ml or 2 ml lobind tubes.

2. Methods

2.1 Sample preparation

Lysis and thiol-reduction

There is no universal protocol to lyse any kind biological material but heating in the presence of high concentrations of SDS and reduction of disulfide bridges usually results in total or at least high extent of lysis.

Examples for total lysate preparation:

50 µl HeLa cell pellet (one ø 15 cm dish, about 2 mg of total proteins), are lysed by mixing with in 500 µl of 1X lysis buffer and incubation at 95°C for 3 min. The DNA has to be sheared by sonication to reduce the viscosity of the sample. Before starting sample processing the lysate has to be clarified by centrifugation at 16,000 x g for 5 min. Quantify your protein extract using BCA kit.

2.2 Sample processing

1. In a lobind tube, mix up to 100 µg of your protein extract with 1X lysis buffer to reach a volume of 92 µl.

2. Add 4 µl of reductant (final concentration 5 mM TCEP) and incubate at 55 °C for 15 min.

3. Add 4 µl of alkylator (final concentration 20 mM ClAA) and incubate at RT for 10 min.

4. Add 15 µl of acidifier to the 100 µl sample (final concentration ~2.5% phosphoric acid). 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 phosphoric acid to reach pH ≤ 1.

5. Add 690 µl of binding/wash buffer to the sample and mix by vortexing (thermomixer, 1250 RPM, 5 minutes at room temperature). Sufficient proteins may make the colloidal protein particulates visible, giving a translucent appearance. All sample – including any resulting colloid – must be transferred to the S-Trap. **Do not centrifuge at this point.**

6. Place an S-Trap micro-column in a 2 ml lobind tube for waste flow through. Apply sample to S-Trap column (400 ul maximum per shot, and centrifuge at 4,000 g for 1 min per shot.). Transfer all sample including anything insoluble into the S-Trap. No column preequilibration is necessary.

7. Add 150 µl binding/wash buffer; centrifuge at 4,000 g for 1 min. Repeat 3 times and discard flow through as necessary. For best results, rotate the S-Trap micro units (like a screw or knob) 180 degrees between the centrifugations of binding and wash steps. This is especially important when using a fixed-angle rotor because the spin column does not experience homogenous flow. A mark on the outside edge during centrifugation makes it easy to track rotations. 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 50% $CHCl_3$ /50% MeOH, filling the entire column each time, then perform three washes with standard methanolic binding/wash buffer as normal. After each centrifugation step, make sure that all added solution has gone through the S-Trap column.

8. Centrifuge S-Trap column at 4,000 g for 1 min to fully remove binding/wash buffer. Failure to do so may result in dripping.

9. Transfer S-Trap micro column to a clean 1.5 ml lobind tube for the digestion.

10. Make a hole in the lid of the column with a small needle (e.g. 21G syringe needle). This step is important because an airtight seal will force the solution out of the S-Trap during incubation. Add 20 µl of digestion buffer containing protease, most commonly trypsin or a trypsin/lys-C mix, at a 1:10 weight to weight (wt:wt) ratio into the top of the S-Trap. By example, for 50 µg of protein to digest, apply 5 µg of trypsin dissolved in 20 µl. Do not apply less than 1 µg of trypsin for effective digestion. 200 mM TEAB is recommended as digestion buffer for trypsin or trypsin/lys-C mixes, but TRIS or ammonium bicarbonate work as well. Visually confirm no air bubbles are present at the top the trap. Bubbles prevent the digestion buffer from entering the trap. If bubbles are present, flick the tube to remove them and/or centrifuge extremely briefly on a bench top centrifuge, returning any flow through to the top of the spin column. The S-Trap is hydrophilic and will absorb the digestion buffer. Careful observation shows that the applied digestion buffer "sinks" by a fraction of a mm when first applied. Do not damage the matrix 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.

11. Add parafilm to prevent evaporation.

12. Incubate overnight at 37 °C. Preferably use a water bath or stationary thermomixer. Ensure the entire column is exposed to heat. Do not shake.

13. Add 60 µl of elution buffer 1 to the S-Trap then centrifuge (4,000 g, 1 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. Transfer the eluate to a new 1.5 ml lobind tube.

14. Add 60 µl of elution buffer 2 to the S-Trap then centrifuge (4,000 g, 1 min). Transfer the eluate to the 1.5 ml lobind tube containing the first eluate.

15. Add 60 µl of elution buffer 3 to the S-Trap then centrifuge (4,000 g, 1 min). This elution assists in recovery of hydrophobic peptides. Other organics may also be used as needed. Transfer the eluate to the 1.5 ml lobind tube containing both the first and second eluates.

16.Speedvac to dryness the pooled eluted peptides.

17. **Add 100 ul (for an IP digestion or 20 µg of digested total extract) to 300 µl (for 50-100 µg of digested total extract) of 1% formic acid (in MS-grade water). Resuspend by vortexing (thermomixer, 1250 RPM, 5 minutes at room temperature). Centrifuge at 17,000 g for 5 minutes. Carefully transfer the supernatant to a new lobind tube (leave some ul at the bottom of the old tube to avoid disruption/pipetting of unsoluble particules). No further C18 cleanup is necessary.**

2.3 Yield determination

Concentration of the peptides can be estimated by Nanodrop absorbance at 205 nm. Dilute your sample to a concentration of **no more than 250 ng/µl** and transfer the entire volume into an MS glass vial.

Notes

1. Remember to wear gloves at all times during the procedure. Do not scratch any part of your body during the procedure. Keratin contamination is less of an issue after peptide digestion, as whole keratin proteins will not interfere with MS analysis.
2. When preparing any stock solutions, avoid using any spatula that has not been cleaned thoroughly with water and ethanol. In fact, we routinely avoid spatulas for weighing/transferring fine powders to prevent contamination and cross-reaction, instead tipping solids directly into clean Eppendorf tubes to maintain purity.
3. Peptides tend to stick to plastic surfaces on storage. We use 1.5 ml lobind tubes that are designed for storing peptides at low concentration.