HYPERsol FFPE protocol with PIXUL megasonication

Required equipment and materials:

- S-Trap 96-well plates
- Costar 3799 96-well plate
- Razor blade and scapula; optionally microtome
- 1 mm biopsy punch; e.g. Kai Biopsy Punch (Miltex-Integra 33−31AA)
- Fine tweezers
- Heat blocks set to 80 °C and 55 °C
- Incubator at 47 °C with water-saturated atmosphere
- PIXUL megasonicator, see www.protifi.com/pixul
- Optionally automation, see www.protifi.com/A200

Required reagents and solutions:

- All standard S-Trap reagents except the lysis buffer (acidification, binding/wash, reduction, alkylation and elution buffers as well as digestion buffer containing protease)
- FFPE samples ideally (**100 – 300 µg**)
- Tris base and hydrochloric acid for pHing
- Sodium dodecyl sulfate (**SDS**)
- LC/MS grade methanol (**MeOH**) and chloroform
- BCA assay e.g. Pierce cat. no. 23225
- In the EU and Japan, an organic solvent such as xylene or methyl tert-butyl ether (**MTBE**)

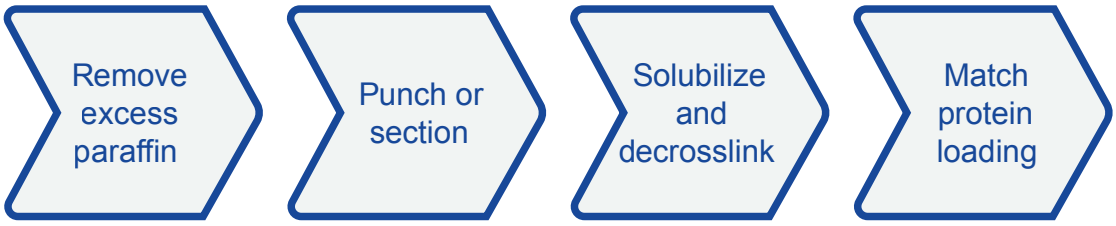
Required solutions (available for separate purchase at www.protifi.com):

Solution	Composition	pH	Storage
HYERsol lysis buffer	10% SDS, 100 mM tris pH 8.5	8.5	1 year at room temp (**RT**)

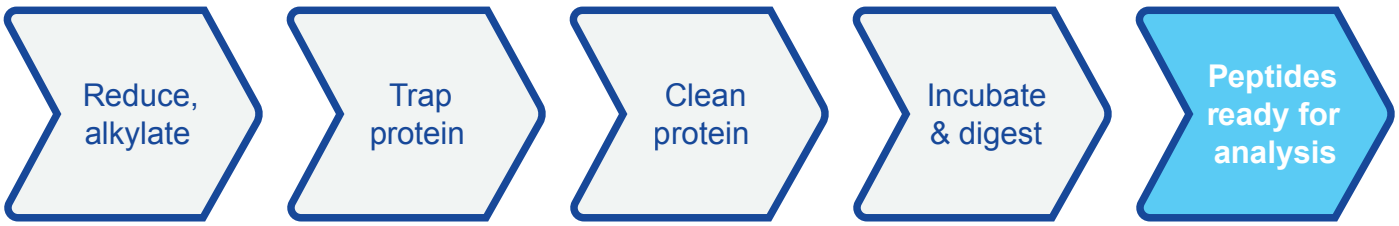
Reference

Marchione DM, Ilieva I, Devins K, Sharpe D, Pappin DJ, Garcia BA, Wilson JP, Wojcik JB. HYPERsol: High-Quality Data from Archival FFPE Tissue for Clinical Proteomics. *Journal of Proteome Research*. 2020 Jan 14;19(2):973-83. https://doi.org/10.1021/acs.jproteome.9b00686

Protocol overview



Subsequent to these steps, follow the standard S-Trap protocol:



Remove excess paraffin

1) Trim FFPE blocks of excess paraffin using a sterile razor blade or scapula.

Punch or section

2) Use a 1 mm Kai Biopsy Punch (Miltex-Integra 33−31AA) to punch cores until 5 mg of total FFPE material is obtained; pool as necessary. Alternatively, section scrolls on a microtome.

Solubilize and decrosslink

3) For tissue cores, preferably dice them into small pieces with a scapula or razor blade and transferred to Costar 3799 96-well plate. For scrolls, transferred it to the 96-well plate and use tweezers to ensure they are at the bottom of the well.

4) In the EU and Japan, completely remove paraffin from the sample with an organic solvent such as xylene or methyl tert-butyl ether (MTBE): add 100 µL, shake samples for 5 min and remove all organic solvent to yield deparaffinized samples. Repeat as necessary to completely remove paraffin.

5) Add 100 µL of HYPERsol lysis buffer or 20X volume/weight if not 5 mg to the wells.

6) Allow samples to rehydrate overnight.

7) Megasonicate at 20 °C for 5 min on the PIXUL or until pieces have been dissolved: 50 N pulse at 1 kHz with a 20 Hz burst rate.

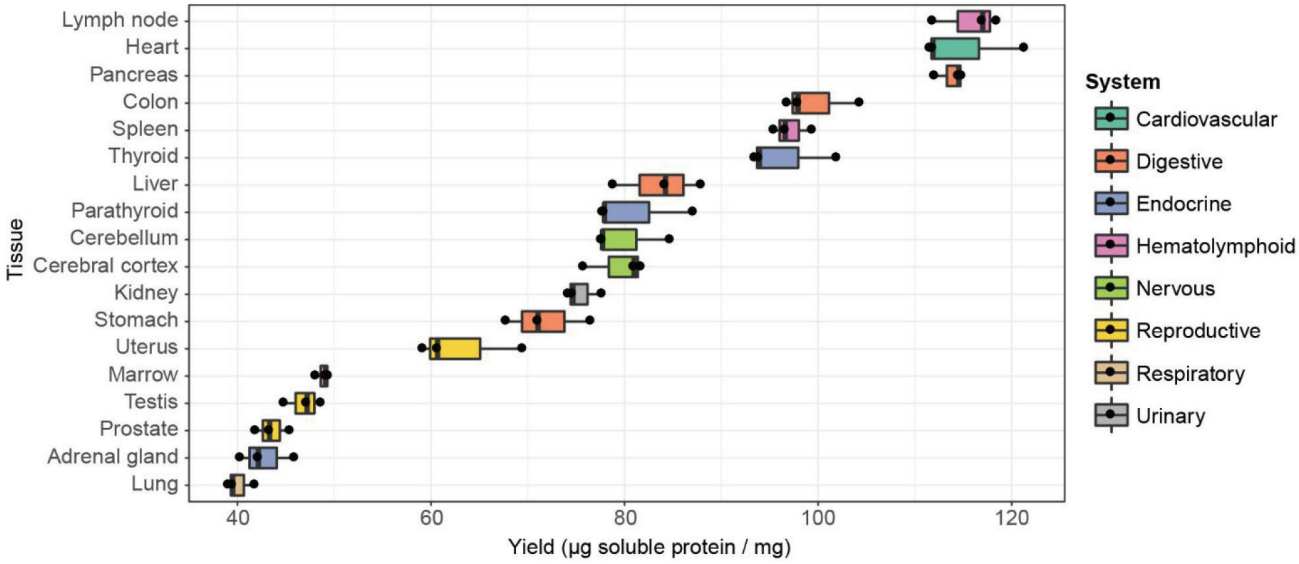
8) Placed on a heat block at 80 °C for 1 h to reverse crosslinking. Make sure to poke small holes in the plate seal to relieve pressure and spin down condensate.

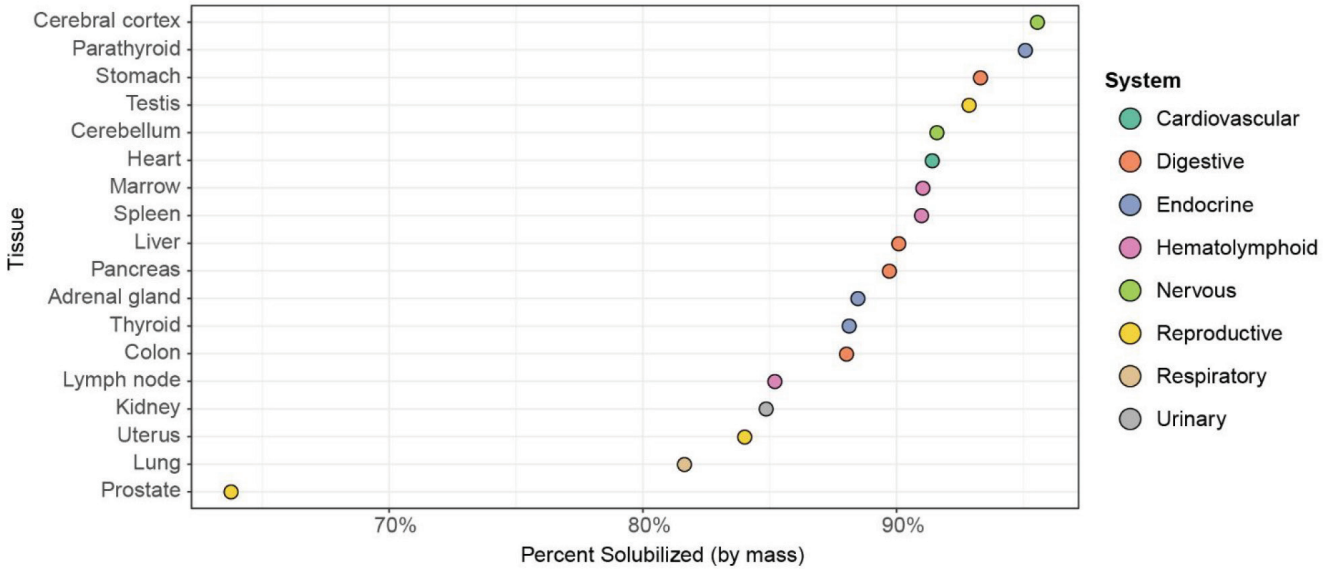
9) Megasonicate for 6 min with the same settings as step 6.

Match protein loading

10) Measure protein concentration with a BCA assay and match the amount of protein in all samples by dilution, as needed, with HYPERsol lysis buffer. 96-well S-Traps process 100 – 300 µg of total protein.

- If samples are very dilute, they can be concentrated as the S-Trap can handle SDS concentrations ≤ 20%.
- For human samples, the following protein yields can be expected (mass in the below graph includes the mass of the wax in the tissue and no external wax):





Abbreviated S-Trap digestion; see also the standard S-Trap protocol

11) Aliquot a fixed quantity of protein from 100 – 300 µg to a clean 96-well plate in a constant volume of 46 µL.

- Samples of different volumes are acceptable if all reagent ratios are maintained.

12) Reduce by adding 2 µL of reductant (120 mM aqueous TCEP; final concentration 5 mM) and incubate at 55 °C for 15 min.

13) Alkylate by adding 2 µL of alkylator (500 mM MMTS in isopropanol; final concentration 20 mM) and incubate at RT for 10 min.

14) Acidify by adding 5 µL of 96-well plate acidifier (27.5% wt/wt aqueous phosphoric acid); mix.

15) Add 350 µL of binding/wash buffer (100 mM final TEAB in 90% MeOH) to all samples. Transfer the sample including any colloidal or precipitated protein to the S-Trap plate and bind via centrifugation, positive or negative pressure. The Tecan A200 is recommended.

16) Wash once with 400 µL of bind/wash buffer.

17) Wash three times with 400 µL of 50% MeOH/50% $CHCl_3$.

- The additional washes ensure that no wax is present.

18) Wash twice more with 400 µL of binding/wash buffer.

19) Add 125 µL of digestion buffer containing sufficient trypsin or trypsin/lys-C mix for a 1:10 wt:wt ratio.

20) Incubate at 47 °C for 1 hr.

21) Remove the plate and add 100 µL of digestion buffer (not containing any additional protease).

22) Incubate at 47 °C for 1 hr more.

23) Elute by centrifugation, positive or negative pressure; the Tecan A200 is recommended.

24) Elute with 80 µL of 0.2% aqueous formic acid followed by 80 µL of 50% aqueous ACN containing 0.2% formic acid. The final ACN concentration will be around 10% v/v. Pool and dry down elutions, resuspending as necessary.