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
- 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

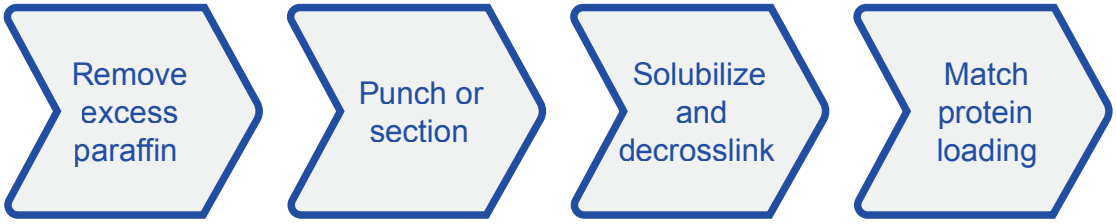
Required solutions (available for separate purchase at www.protifi.com):

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

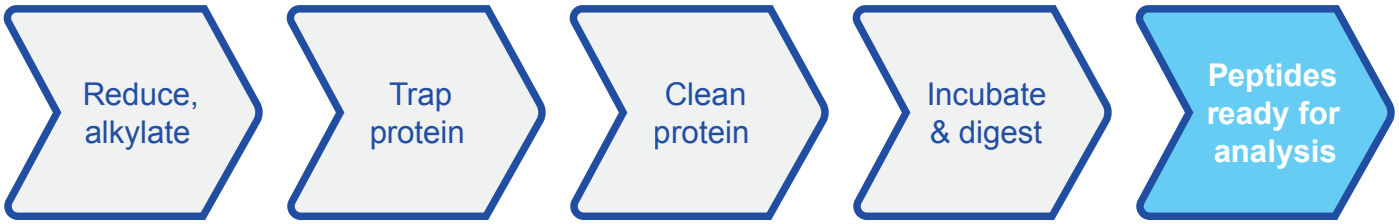
Reference

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

5) Allow samples to rehydrate overnight.

6) 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.

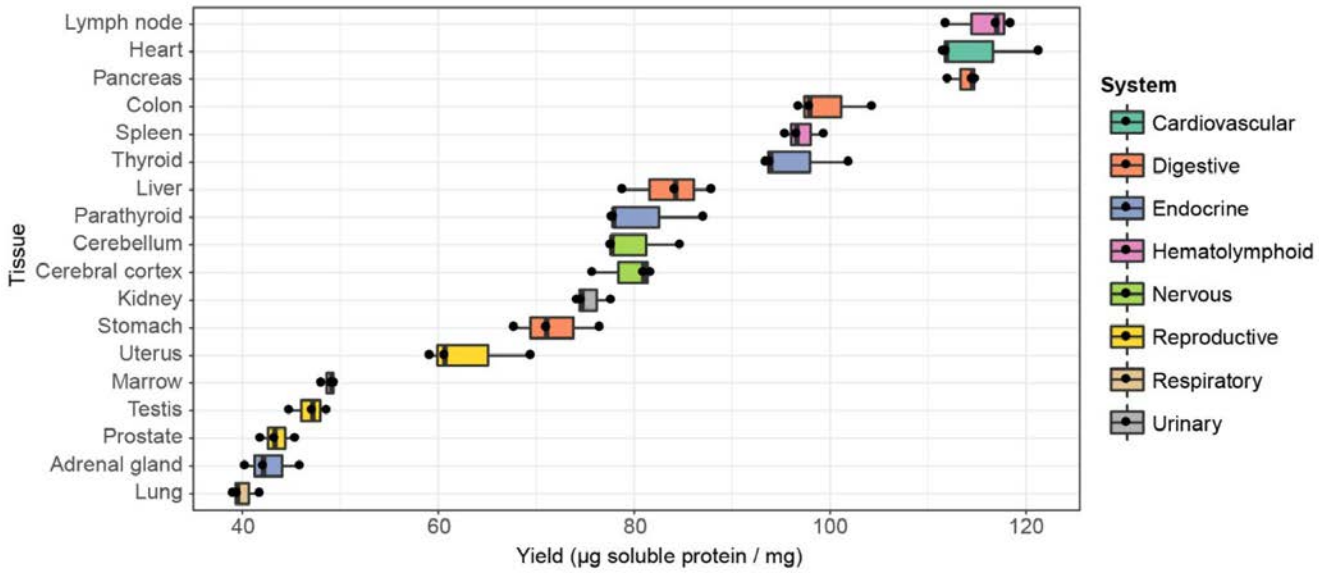
7) Placed on a heat block at 80 °C for 1 h to reverse crosslinking. Make sure to poke small holes in the plate seal to relieve pressure and spin down condensate.

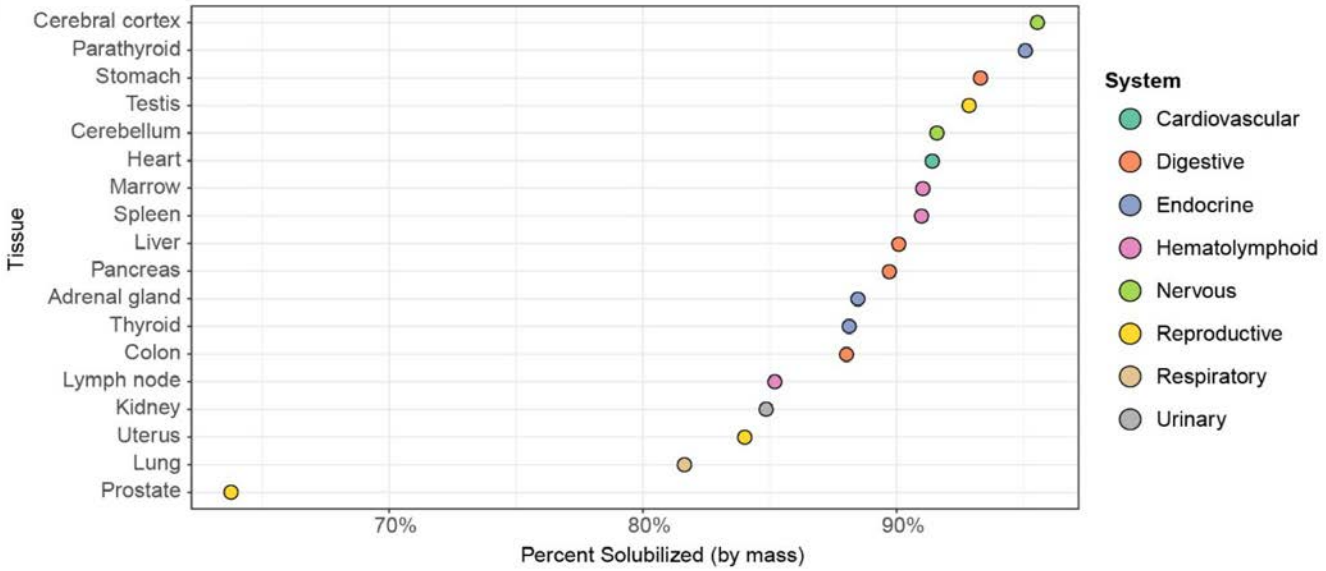
8) Megasonicate for 6 min with the same settings as step 6.

Match protein loading

9) 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

10) 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.

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

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

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

14) 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.

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

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

- The additional washes ensure that no wax is present.

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

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

19) Incubate at 47 °C for 1 hr.

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

21) Incubate at 47 °C for 1 hr more.

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

23) 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.