HYPERsol: flash-frozen results from archival FFPE samples for clinical proteomics

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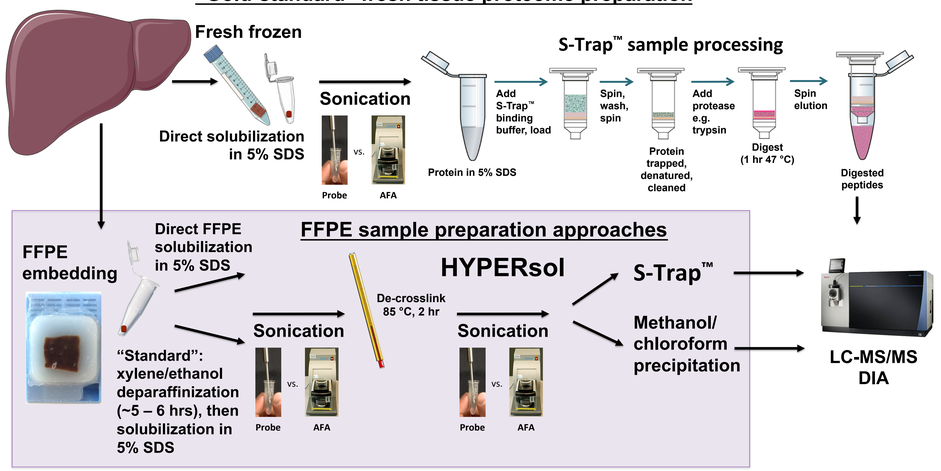
Abstract

- Formalin fixed paraffin embedding (FFPE) is a decades-old sample preparation technique common in experimental research and medicine. FFPE samples can be stored indefinitely at room temperature, resulting in an exceptionally large and rich worldwide collection. Despite its potential to significantly impact medicine, proteomic analysis of FFPE samples has lagged. Traditionally, samples are first laboriously deparaffinized with often-toxic organic solvents. Subsequent protein extraction is extremely critical but no consensus has been reached as to an optimal protocol.
- Here we present **H**igh-**Y**ield **P**rotein **E**xtraction and **R**ecovery by direct **SOL**ubilization (HYPERsol), a standardized one-pot workflow for FFPE proteomics. The HYPERsol workflow combines the HYPERsol buffer (5% SDS), Covaris AFA ultrasonication and ProtiFi S-Trap sample processing. HYPERsol eliminates deparaffinization and exhaustively solubilizes entire FFPE samples, including the paraffin, yielding ID rates and quantification values very similar to flash-frozen (**R = 0.94**).
- To benchmark HYPERsol performance, we compared tissue treated in parallel either by flash-freezing or FFPE according to standard histopathology procedures. All samples were extracted with SDS using probe sonication or Covaris AFA ultrasonication. SDS was removed by standard precipitation or S-Traps. Protein identification rates and reproducibility were evaluated after analysis on a Thermo QE HF-X or Fusion mass spectrometer.
- Compared to standard procedures, the use of S-Traps resulted in significant increases in peptide (≥30%) and protein identification rate (≥20% increase) with greater reproducibility. The use of AFA decreased hands-on time, increased ID rates an additional 6% – 8% and significantly increased protein yield from FFPE samples (80% – ≥200%). The HYPERSol combination yielded ID rates comparable to those obtained from fresh frozen tissue (101%/ 97% ID rate for peptides/proteins) while eliminating toxic xylene and saving approximately 5 – 6 hrs in sample processing by eliminating organic deparaffinization.
- HYPERSol solves the problem of extraction bias and achieves the goal of reproducible standardized protein recovery from FFPE samples in a workflow suited to automated, high-throughput analyses. We anticipate this workflow will assist to usher in a new era of clinical proteomics.

Workflow/Method

"Gold-standard" fresh tissue proteome preparation

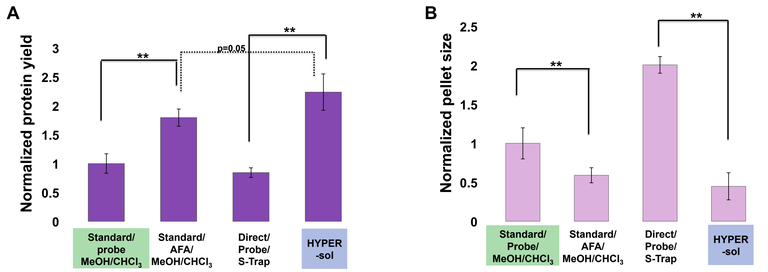


Schematic depiction of the proteomic workflow comparing, from the same biospecimen, FFPE sample preparation to the "gold-standard" of preparation from fresh tissue. The sample was immediately split at the time of tissue collection. Half was frozen and half was placed in a cassette, fixed in formalin and processed according to standard histopathology protocols. FFPE sample treatment is highlighted in the purple box. 1 mm tissue cores from FFPE blocks were either placed directly in buffer containing 5% SDS, or subjected to standard deparaffinization by incubations in xylene and a graded concentration series of aqueous ethanol. Wax-free tissue was subjected to homogenization and protein solubilization in 5% SDS buffer. Samples were homogenized with a mortar and pestle and passed through syringe needles before (ultra)sonication either using a bench-top probe sonicator or a Covaris S220 focused ultrasonication system. Samples were decrosslinked for 2 hrs at 85 °C then subjected to a second (ultra)sonication step with the same method as the first sonication. Finally, SDS was removed from the samples using either methanol-chloroform precipitation or an S-Trap (ProtiFi, LLC, www.protifi.com). Following reduction and capping of disulfides, trypsin digestion were conducted either in-solution for MeOH/$CHCl_3$ samples or in S-Traps. All samples were subjected to LC-MS/MS analysis using the same gradient and method on a Fusion Tribrid or QE-HFX mass spectrometer using data-independent acquisition. The data were searched against a database consisting of peptides identified in a fractionated pool (6 fractions) of all samples run on the same column in DDA mode and the direct DIA identifications from the pooled runs.

Sample preparation step	Conditions tested
Deparaffinization	• Standard deparaffinization *vs.* • Direct dissolution
Tissue homogenization/ extraction	• Benchtop probe sonicator *vs.* • Covaris AFA ultrasonication
MS sample preparation	• S-Trap *vs.* • Precipitation and in-solution digestion

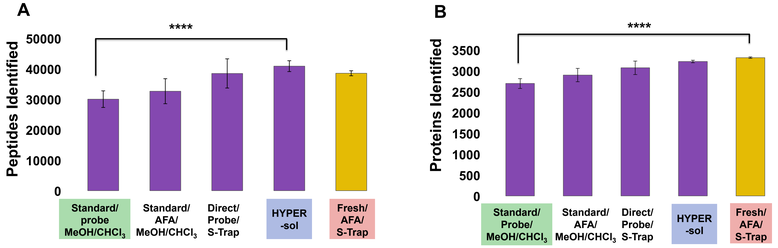
Results

1) HYPERsol markedly increases FFPE protein yield and corresponding decreases pellet size



Normalized total protein yield **(A)** from four biological replicates of FFPE tissue samples processed according to the listed parameters for deparaffinization, sonication/extraction and SDS removal/digestion/purification. The corresponding normalized pellet weights **(B)** from the same samples. The HYPERsol combination of direct solubilization in the HYPERsol buffer followed by AFA protein extraction significantly increases protein yields by enhancing solubilization of FFPE samples. Error bars are +/- standard deviation; p-values are from two-tailed t-tests.

2) The HYPERsol S-Trap/AFA FFPE protocol improves peptide/protein ID rates and reproducibility

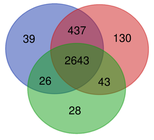


- Total number of proteins (**A**) and peptides (**B**) identified in DIA LC-MS/MS analysis of protein samples processed according to the listed parameters for deparaffinization, sonication/extraction and SDS removal/digestion/purification. Data is shown for eight replicates of human liver tissue prepared from FFPE samples, and is compared to the number of peptide/protein identifications from fresh-frozen liver tissue processed in parallel (gold bars, highlighted in red). Our lab's previous protocol that corresponds to a typical FFPE preparation scheme is highlighted in green. The optimized S-Trap/AFA FFPE extraction protocol is highlighted in blue. Error bars are +/- standard deviation; p-values are from two-tailed t-test.
- The optimized HYPERSol protocol improved ID rates and simultaneously, as judged by similarity of peptides and proteins identified across samples, increased reproducibility. The Venn diagrams and tables below demonstrate this improved reproducibility with the number of total peptides **(C)** and proteins **(D)** identified in both of two separate sample preparations using the old (green) and new, optimized HYPERsol FFPE processing method (blue) as compared to the gold-standard extraction from fresh frozen tissue (red).

C Peptide ID

		Deparaffinization	Homoge-nization/ extraction	Sample prep	IDs, both replicates		% of total Identified
Red	N/A (fresh frozen)		AFA	S-Trap	Peptide	35686	78.7%
					Protein	3253	97.2%
Blue	HYPERsol: Direct with SDS		AFA	S-Trap	Peptide	35244	77.8%
					Protein	3145	93.4%
Green	Standard (xylene/ EtOH)		Probe	Precipitation, in-solution digestion	Peptide	28221	62.3%
					Protein	2740	81.9%

D Protein ID



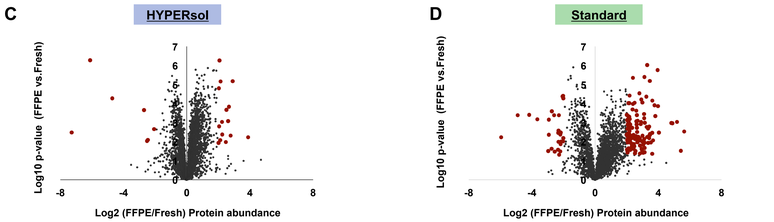
3) Protein/peptide quantification from optimized FFPE protocol more closely reflect those from fresh tissue

A Peptide Quantification

	Direct/probe/ S-Trap	Direct/AFA/ S-Trap	Fresh/probe/ S-Trap	Fresh/AFA/ S-Trap	Standard/AFA/ S-Trap	Standard/probe/ MeOH/CHCl3	Standard/AFA/ MeOH/CHCl3
Direct/probe/ S-Trap	1.00	0.96	0.93	0.92	0.87	0.87	0.88
	0.96	1.00	0.93	0.94	0.86	0.88	0.87
Direct/AFA/ S-Trap	0.93	0.93	1.00	0.98	0.92	0.93	0.93
	0.92	0.94	0.98	1.00	0.91	0.94	0.93
Fresh/probe/ S-Trap	0.87	0.86	0.92	0.91	1.00	0.97	0.98
	0.87	0.88	0.93	0.94	0.97	1.00	0.97
Fresh/AFA/ S-Trap	0.88	0.87	0.93	0.93	0.98	0.97	1.00
	0.88	0.87	0.93	0.93	0.98	0.97	0.99
Standard/AFA/ S-Trap	0.95	0.96	0.95	0.96	0.89	0.90	0.91
	0.94	0.95	0.96	0.97	0.90	0.91	0.92
Standard/probe/ MeOH/CHCl3	0.76	0.76	0.78	0.76	0.73	0.73	0.74
	0.82	0.81	0.84	0.82	0.78	0.77	0.79
Standard/AFA/ MeOH/CHCl3	0.73	0.73	0.77	0.76	0.71	0.72	0.73
	0.76	0.76	0.80	0.78	0.74	0.75	0.76

B Protein Quantification

	Direct/probe/ S-Trap	Direct/AFA/ S-Trap	Fresh/probe/ S-Trap	Fresh/AFA/ S-Trap	Standard/AFA/ S-Trap	Standard/probe/ MeOH/CHCl3	Standard/AFA/ MeOH/CHCl3
Direct/probe/ S-Trap	1.00	0.95	0.91	0.90	0.87	0.85	0.86
	0.95	1.00	0.89	0.89	0.84	0.84	0.84
Direct/AFA/ S-Trap	0.91	0.89	1.00	0.96	0.93	0.92	0.93
	0.90	0.89	0.96	1.00	0.93	0.93	0.94
Fresh/probe/ S-Trap	0.87	0.84	0.93	0.93	1.00	0.97	0.98
	0.85	0.84	0.92	0.93	0.97	1.00	0.97
Fresh/AFA/ S-Trap	0.86	0.84	0.93	0.94	0.98	0.97	1.00
	0.86	0.84	0.93	0.94	0.98	0.97	0.98
Standard/AFA/ S-Trap	0.92	0.92	0.94	0.95	0.92	0.92	0.92
	0.92	0.92	0.94	0.95	0.92	0.92	0.92
Standard/probe/ MeOH/CHCl3	0.86	0.84	0.89	0.87	0.85	0.84	0.85
	0.82	0.81	0.84	0.82	0.78	0.77	0.79
Standard/AFA/ MeOH/CHCl3	0.83	0.82	0.89	0.88	0.87	0.85	0.86
	0.76	0.76	0.80	0.78	0.74	0.75	0.76



Pearson correlation coefficients for normalized peptide (**A**) and protein (**B**) intensities between the specified sample preparation conditions. The center box (light black light line) contains sample preparation conditions in which protein and peptide quantification correlations average >0.90 across replicates; all employ S-Trap sample processing. Quantifications from HYPERsol FFPE sample preparation using AFA and S-Traps (blue highlight of labels) correlate well with measurements from fresh frozen tissue (red label highlight at left and top; average R = 0.94) at both the peptide and protein levels. Other methods produce less uniform and complete extractions, like that previously employed by our lab (purple box highlight) and show mostly stable quantifications among themselves (light black box at lower right) but significantly less agreement with fresh tissue processed in parallel (R = 0.73 – 0.79 and 0.82 – 0.89 for peptides and proteins, respectively, purple boxes). The volcano plots **(C)** and **(D)** depict the ratio of protein abundances measured in FFPE compared to fresh tissue from three replicate preparations of each sample. Using the optimized HYPERsol protocol, only 23 proteins of 3206 (0.7%) show significantly increased or decreased expression in FFPE as compared to fresh tissue ($p < 0.05$; $\log_2$ increased/decreased abundance < 2). By contrast, standard sample preparation workflows lead to a greater number of proteins showing significant, spurious changes in abundance caused by sample processing (146 proteins of 2845, 5.1%).

Conclusions

- The HYPERsol combination of 5% SDS, AFA ultrasonication and ProtiFi S-Traps markedly increases protein yield from FFPE samples. We anticipate this will facilitate analysis of small samples such as clinical needle biopsies.
- HYPERsol is rapid and obviates the need for time-consuming, tedious and toxic deparaffinization. Samples can now be easily prepared to peptides and analyzed on the same day.
- The optimized HYPEsol workflow leads to reproducible identifications and quantifications of proteins and peptides at rates very similar to those obtained from fresh tissue (**R = 0.94**).
- We anticipate that the reduced variability of sample processing afforded by HYPERsol will enhance our ability to extract meaningful biologic information from FFPE datasets. This includes the ability to now distinguish original sample quality from artifacts induced by sample preparation.
- HYPERsol solves the problem of extraction bias and achieves the goal of reproducible standardized protein recovery from FFPE samples. The workflow is suited to automated, high-throughput analyses including 96-well plate formats essential for clinical implementation. We anticipate HYPERsol will assist to usher in a new era of clinical proteomics.

Poster QR

This work is available as a bioRxiv preprint as "HYPER-sol: flash-frozen results from archival FFPE tissue for clinical proteomics" at https://www.biorxiv.org/content/10.1101/632315v1.full.

Preprint paper QR