Total solubilization of FFPE samples for high throughput 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, the utility of FFPE remains largely unexplored. The workflow for 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.

We present a one-pot solution which eliminates deparaffinization and employs 5% SDS and S-Trap sample processing to exhaustively solubilize entire FFPE samples. To benchmark the performance of this method, we compared flash-frozen and FFPE tissue treated according to standard histopathology procedures. All samples were extracted with SDS using standard techniques (pulverization, syringe needles), probe sonication or Covaris AFA ultrasonication. SDS was removed by standard precipitation of S-Traps. Protein identification rates and reproducibility were evaluated after analysis on a Thermo Q HFX or Fusion mass spectrometer.

Compared to standard procedures, the use of S-Traps resulted in significant increases in peptide (33%) and protein identification rate (22%) 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% ~ 220%). The combination of S-Trap and AFA yielded ID rates comparable to those obtained from fresh frozen tissue (101% 0% ID rate for peptides and proteins) while eliminating toxic xylene and saving approximately 5 - 6 h in sample processing by eliminating deparaffinization.

Our system 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

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 alcohol/ether. 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 (ultraspin) or either using a bench-top sonicator or a Covaris S2200 focused ultrasonication system. Samples were decolorized at 2 hour for 86% but then subjected to a second (ultraspin) step with the same method as the first sonication. Finally, SDS was removed from the samples using either methanol/chloroform precipitation or an S-Trappolymer, LLC www.protif.com. Following reduction and alkylation of disulfide linkages, digested peptide were either run in solution for MS or in S-Traps. All samples were subjected to LC-MS/MS analysis using the same gradient and method on a Fusion Triblend or Q-Exactive mass spectrometer using data-independent acquisition. The data were searched against a database consisting of peptides identified in a fosmid pool of (B fraction) of all samples run on the same column in DQA mode and the direct DIA identifications from the pooled runs.

Results

1) 5% SDS with AFA sonication markedly increases FFPE protein yield and corresponding decreases pellet size

Normolized total protein yield (A) from four biological replicates of FFPE tissue samples processed according to the listed parameters for deparaffination, sonication/extraction and SDS removal/digestion/purification. The corresponding normalized pellet weights (B) from the same samples. The combination of direct sonication in 5% SDS 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) Optimized S-Trapp/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 deparaffination, 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 tissue processed in parallel (gold bars, highlighted in red). Our lab’s previous protocol that corresponds to the typical FFPE preparation scheme is highlighted in green. The optimized S-Trapp/AFA FFPE extraction protocol is highlighted in blue. Error bars are +/- standard deviation; p-values are from two-tailed t-test.

The optimized protocol improved ID rates and simultaneously, as judged by similarity of peptide and protein IDs identified across samples, increased reproducibility. The diagrams demonstrate the number of total peptides (C) and proteins (D) identified in both of two separate sample preparations using the old (green) and new, optimized FFPE processing method (blue) as compared to the gold-standard extraction from fresh tissue (red).

Conclusions

- The 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.
- The combination of S-Trapp/AFA FFPE protocol is robust and obviates the need for time-consuming, tedious deparaffinization steps, and is easily adopted to clinical practice.
- The optimized workflow using S-Traps, 5% SDS and AFA leads to reproducible identifications and quantifications of proteins and the peptides at rates similar to those obtained from fresh tissue.
- The peptide and protein expression patterns generated by our protocol will enhance our ability to extract meaningful biologic information from FFPE databases. This includes the ability to now distinguish original sample quality from artifacts induced by sample preparation.
- Our system solves the problem of extraction bias and achieves the goal of reproducible standardized protein recovery from FFPE samples. This workflow is suited to automated, high-throughput analyses including 96-well plate formats essential for clinical implementation. We anticipate this workflow will assist to usher in a new era of clinical proteomics.