Universal Sample Processing Of Multiple Sample Types For Reproducible Proteomic Sample Preparation

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1) Introduction and method

- Proteomics analyses typically begin with sample lysis and protein extraction. This single step is responsible for the vast majority of variability in proteomics data (Fig. 1). This variability has limited the utility of proteomics and its application in clinical settings.
- Variability in sample extraction arises predominantly from two sources: first, samples can experience different levels of physical force (disruption), which results in different levels of protein extraction; and second, the difficulty of reproducibly dissolving proteins with extremely diverse solubility properties.
- Here, we employ 5% SDS as a universal protein solvent, Covaris AFA* technology for sample disruption and extraction and ProtiFi S-Traps* to capture, concentrate and clean proteins from 5% SDS. By imparting strong controlled acoustic forces, AFA reproducibly homogenizes samples and forces proteins completely into solution. ProtiFi S-Trap sample processing then rapidly concentrates the proteins, cleans them of contaminants and detergents, and digests them in-column.
- This combined workflow is universal: the solubilization power of 5% SDS with the extreme sheer forces afforded by AFA is unrivaled and sufficient to reproducibly and fully extract proteins from all sample types from hard tissues to cell cultures without change.
- The solubilizing power of this workflow is sufficient to fully dissolve FFPE blocks in aqueous 5% SDS; S-Trap sample processing then fully removes the SDS-solubilized paraffin and samples are processed without change to the S-Trap protocol.

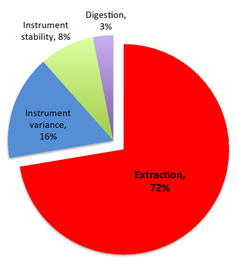


Fig. 1: The vast majority of variability in proteomics data arises during protein extraction. Percent of total variability. Data from reference 1.

2) Covaris Adaptive Focused Acoustics (AFA) technology

The Adaptive Focused Acoustics (AFA) technology[2] is a unique process to deliver controlled, non-contact sonic energy to isolated, biological samples while maintaining iso-thermal conditions. Unlike regular sonicators, which operate at lower frequencies (e.g, 20 kHz) and a long wavelength (~10 cm in water), the Covaris acoustic transducer operates at higher frequencies (e.g. 1 MHz) which results in correspondingly shorter wavelengths (~3 mm in water). This combination of high frequency and converged energy enable precise, efficient control of sonic energy delivery versus standard sonication techniques (Fig. 1). AFA is produced by a dish-shaped transducer which focuses acoustic energy waves into a small localized high-pressure zone surrounded by a low-pressure field. The very high-speed pressure fluxes create intense sheer forces and turbulent mixing which benefit both rapid heat transfer and rapid solvent boundary layer exchange (Fig. 2). The AFA process enables biological samples to be fully disaggregated and solubilized. Additionally, AFA is an isothermal technique which allows samples to be processed at a constant, predefined temperature (e.g., 4 °C +/- 0.5 °C).

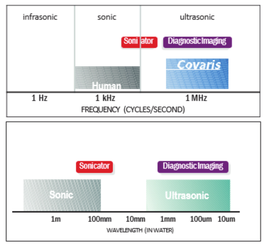


Fig. 2: AFA produces much higher frequencies than standard sonication techniques.

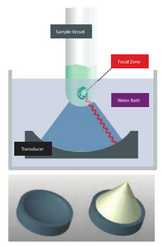


Fig. 3: In AFA, forces converge on a localized area to create intense sheer forces and turbulent mixing.

3) ProtiFi™ S-Trap™ sample processing technology

The Suspension-Trapping™ or S-Trap™[3-5] method is a technique to extract, solubilize and handle all proteins in high concentrations of sodium dodecyl sulfate (SDS, ≤ 15%) prior to their capture, concentration and cleaning and digestion. Proteins are captured in the submicron pores of the S-Trap™ with extremely high surface area to volume ratios. This allows them to be rapidly cleaned of SDS and contaminants including all detergents, urea, salts, glycerol, PEG, Laemmli loading buffer, bile salts, etc. Proteases are then introduced into the pores where tight physically confinement greatly enhances protease-substrate interaction and thus proteolytic activity. Rapid (< 1 hr), reactor-type digestion follows. Capture of protein within the trap (SDS depletion, wash and protease addition) requires just minutes. After a one-hour digest at 47 °C, peptides are eluted and ready for downstream processing.

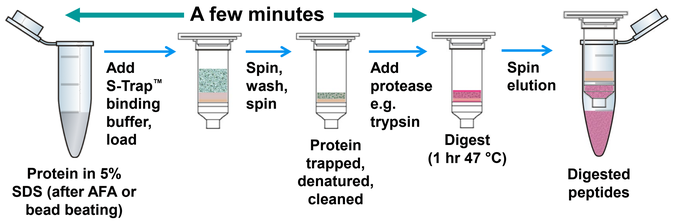


Fig. 4: Steps of S-Trap™ sample processing.

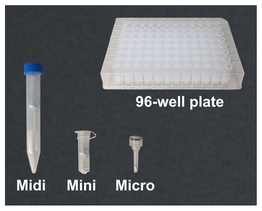
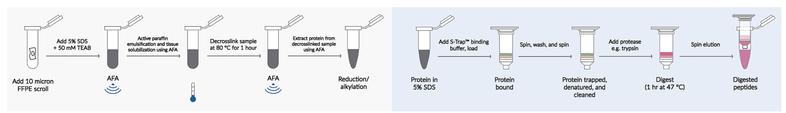


Fig. 5: Available formats of S-Traps™. Micros handle < 100 µg, minis and the 96-well plate 100 – 300 µg and midis > 300 µg. Midis are frequently employed in enrichments including PTM analysis and SISCAPA.

4) Materials and methods

Mouse tissues were collected in IRB approved facilities from male 14-week old black 6 mice. After anesthetization with isoflurane, exsanguination and sacrifice by cardiac puncture, pancreas, brain, liver, kidney, heart, muscle, skin and bone were harvested in that order and immediately frozen in liquid nitrogen. Tissue samples were kept at – 80 °C until use. Human kidney FFPE blocks were obtained from the Cooperative Human Tissue Network (CHTN, www.chtn.org). Briefly, tissue was placed into plastic histology cassettes and covered with 10% neutral buffered formalin (NBF) for 24 hours. Samples were subsequently transferred to 70% ethanol and placed on a tissue processor where serial dehydrations with increasing concentrations of ethanol, then xylene were performed followed by impregnation by paraffin. The FFPE block was sectioned by microtome at 10 um thickness. AFA consumables were obtained from Covaris, Inc. (Woburn, MA; www.covaris.com). S-Traps were obtained from ProtiFi, LLC (Huntington, NY; www.protifi.com). Hard tissues were first pulverized with a Covaris cryoPREP which crushes samples at −196 °C to a fine powder. In general, 5 – 30 mg of tissue were added to 130 µL of extraction buffer and subjected to immediate AFA processing in microTUBE130 tubes (Covaris). AFA was performed on a S220 instrument with peak incident power set to 175 W using 200 cycles per burst and a 10% duty factor at 20 °C for 6 minutes. As an alternative to AFA, a Fast Prep FP120 bead beater was also employed (Savant; **BB** means "bead beater" below; 250 µL extraction buffer buffer, 6 minutes at speed 6.5). Particulate was removed from samples using a 0.2 um nylon spin filter. S-Trap lysis buffer is 5% SDS, 50 mM TEAB pH 7.55. **TP** (total protein) buffer is 7 M urea, 2 M thiourea and 1% CHAPS; other extraction buffers included 125 mM ammonium bicarbonate (no pH adjustment) and 80% 125 mM ammonium bicarbonate/20% acetonitrile. Each protein extraction condition was performed with a minimum of three replicates. Protein concentrations were determined by BCA. S-Trap sample processing was in accordance with the manufacturer's directions. Briefly, SDS was added to 5% (final) to any sample which did not contain SDS. This solution was acidified by addition of phosphoric acid, combined with methanolic S-Trap binding/wash buffer, the proteins were captured on S-Trap micros and thrice washed of detergent and other contaminants with S-Trap wash buffer. Especially fatty tissues (bone marrow) were additionally washed with chloroform to remove lipids. Trypsin (Pierce) was added and digestions were either for 1 hr at 47 °C (1:25) or overnight at 37 °C (1:50). FFPE samples were extracted according to the workflow diagrammed below: AFA in 5% SDS, reverse crosslinking at 80 °C, AFA then S-Trap sample processing. Paraffin is fully dissolved by 5% SDS, heat and AFA, does not interfere with S-Trap sample processing and is fully removed by the normal S-Trap process. Unless otherwise specified, 1 µg of samples were analyzed on a Fusion (Thermo Fisher Scientific; orbi/orbi, 120k, 30k; top N, 3 sec across 130 min gradients at 300 nL/min; UltiMate 3000 Nano LC with an Acclaim PepMap RSLC 2 µm C18 column, 75µm id x 25 cm length). Data were searched with Mascot to a 1% FDR. Note that identification of certain commercial equipment, instruments, software or materials does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for the purpose.



SDS/AFA/S-Trap workflow for FFPE samples.

5) Extraction conditions effect on protein yield

We compared different extraction buffers using AFA and bead beating on both fresh frozen tissues (Fig. 6) and FFPE blocks (Fig. 7), 5% SDS applied with AFA consistently produced the highest protein yields; typically 3x – 8x more was extracted with AFA and SDS than other extraction conditions. Coefficients of variance (CVs) of AFA/SDS extraction were consistently < 10 % (5Ŧ ± 3Ŧ) and significantly lower than TP buffer (Fig. 8). AFA was more reproducible than BB and could be performed in a 96-well plate for transfer via a filter plate to a 96-well S-Trap sample processing plate (Fig. 5).

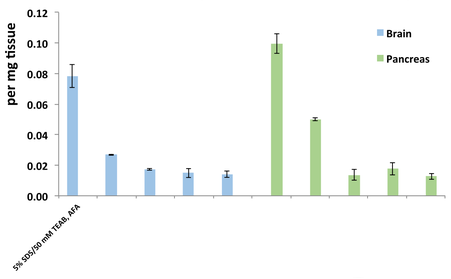


Fig. 6 (left): 5% SDS and AFA consistently resulted in the highest protein yield for fresh-frozen tissue samples.

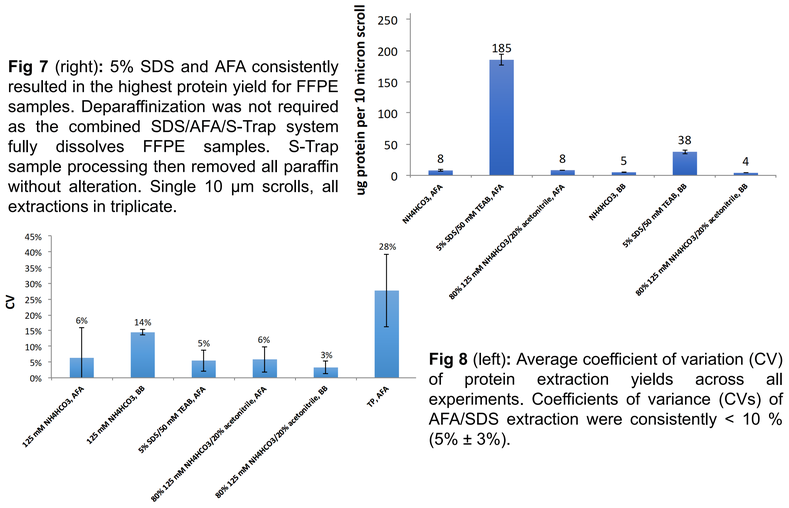


Fig 7 (right): 5% SDS and AFA consistently resulted in the highest protein yield for FFPE samples. Deparaffinization was not required as the combined SDS/AFA/S-Trap system fully dissolves FFPE samples. S-Trap sample processing then removed all paraffin without alteration. Single 10 µm scrolls, all extractions in triplicate.

Fig 8 (left): Average coefficient of variation (CV) of protein extraction yields across all experiments. Coefficients of variance (CVs) of AFA/SDS extraction were consistently < 10 % (5% ± 3%).

6) Extraction conditions effect on protein identification

While aqueous buffers are often preferred in proteomics due to the traditional difficulty of detergent removal, detergents (5% SDS or TP) were absolutely necessary to reliably extract and identify membrane proteins; they also improved ID rates of those from the nucleus and cytoplasm. Membrane protein ID from aqueous buffers was >50% less in brain and fell to zero in pancreas. While biochemically unsurprising, this significant reduction in extracted – and thus observed – proteins calls into question a large number of workflows and what they may have been unable to observe. Note that the yield with TP, a very common buffer for 2D gel work, was at least 5x lower than with 5% SDS (Figs. 6).

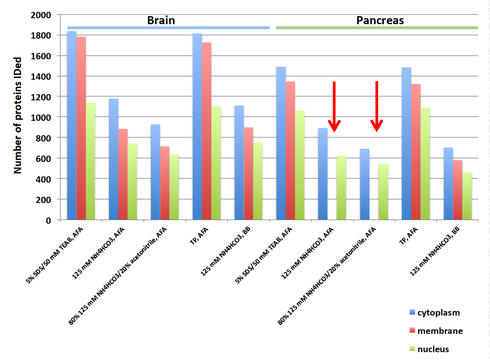
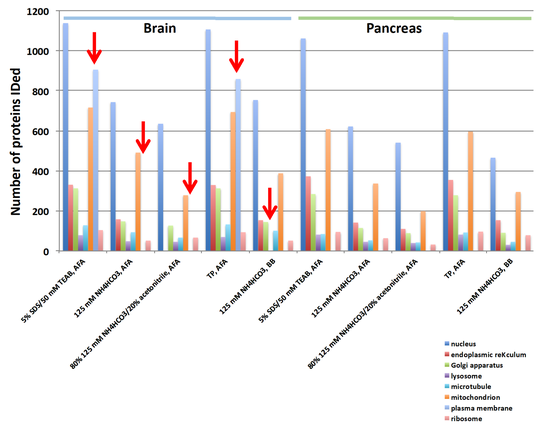


Fig 9: Gene Ontology classification of proteins identified from brain and pancreas as a function of extraction condition. Note the red arrows. Protein content was equally matched prior to S-Trap sample processing.

6) Extraction conditions and protein identification (cont.)

Fig. 10: Cellular organelle as a function of extraction condition. Note the precipitous drop of protein identifications in the absence of detergent. Red arrows indicate proteins identified which localize to the plasma membrane. 905 such proteins were identified with 5% SDS; this falls to zero without detergent. Cf. also orange bars, mitochondria. As biology flows largely, if not fully, through membranous systems, these results suggest harsh protein extraction and solubilization with AFA and 5% SDS followed by S-Trap sample processing is necessary to fully observe biological processes especially membrane-associated processes.



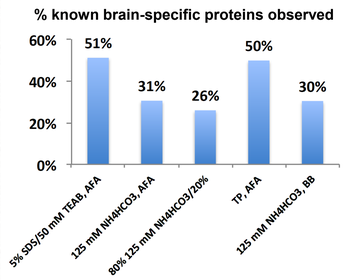


Fig. 11: To gain a sense of the extent of biology missed when detergents are omitted during proteomics sample preparation, we determined the proportion of known brain-specific proteins identified as a function of extraction condition. Of the 432 proteins known to be expressed in the brain with high specificity (Human Protein Atlas, www.proteinatlas.org), approximately half were identified in a single 1D run when detergents were used. This fell to a third to a fourth in their absence. ID rates of brain-specific proteins would be even lower if protein levels had not been matched (cf. Figs. 6 and 14). As the brain is a lipid organ which functions in large part through membrane proteins, these results are likely representative of the observation of other membrane-bound and –associated processes.

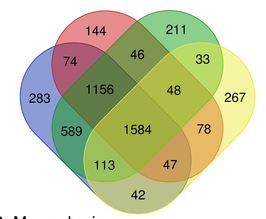


Fig. 12: Mouse brain.

	Extraction buffer	Extraction technique	Digestion technique	Total IDed proteins	% of proteins IDed in any condition
Blue	SDS	AFA	S-Trap	3887	82.5%
Red	NH_4HCO_3	AFA	S-Trap	3779	80.2%
Green	SDS	BB	S-Trap	3276	67.4%
Yellow	NH_4HCO_3	BB	S-Trap	2211	46.9%

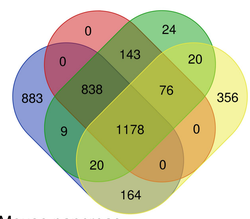


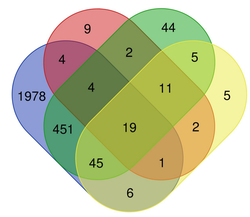
Fig. 13: Mouse pancreas.

	Extraction buffer	Extraction technique	Digestion technique	Total IDed proteins	% of proteins IDed in any condition
Blue	SDS	AFA	S-Trap	3091	83.3%
Red	NH_4HCO_3	AFA	S-Trap	2307	62.2%
Green	SDS	BB	S-Trap	2234	60.2%
Yellow	NH_4HCO_3	BB	S-Trap	1813	48.9%

Mouse brain (Fig.12), pancreas (Fig. 13) or human kidney FFPE scrolls (Fig. 14) were processed either with 125 mM ammonium bicarbonate or 5% SDS. Protein levels were matched before digestion for mouse tissues; for FFPE scrolls a fixed 0.5% of the total extraction was analyzed. 5% SDS with AFA treatment consistently yielded the highest number of identifications in S-Trap sample processing. In Figs. 12 and 13, two 1D runs with that condition were sufficient to identify >80% of the proteins identifiable in all conditions (8 runs in total). For a fixed 0.5% of sample extracted from FFPE, 97% of all protein IDs were obtained with 5% SDS, AFA and S-Traps in a single 1D run.

Fig. 14: Human kidney, 10 um FFPE scrolls.

	Extraction buffer	Extraction technique	Digestion technique	Total IDed proteins	% of proteins IDed in any condition
Blue	SDS	AFA	S-Trap	2508	97.0%
Red	NH_4HCO_3	AFA	S-Trap	52	2.0%
Green	SDS	BB	S-Trap	581	22.5%
Yellow	NH_4HCO_3	BB	S-Trap	94	3.6%



7) Conclusions

- The combination of 5% SDS, AFA and S-Traps is a universal protein extraction, handling and digestion solution which makes sample-specific optimization obsolete.
- 5% SDS with AFA extraction and S-Trap proteomics preparation reproducibly samples the entire proteome and consistently identifies the highest number of proteins, even when protein loading is matched before processing to peptides.
- The combined system is fully suited to high-throughput automation with 96-well plates.
- When applied to FFPE samples, the SDS/AFA/S-Trap solution is a one tube, one column solution which eliminates the need for slow and toxic deparaffinization steps. It significantly increases efficiency, throughput, protein yield and thus protein ID rates.
- We anticipate the combined workflow of Covaris AFA and ProtiFi S-Trap sample processing will enable reproducibility in bottom-up proteomics and thus support the translation of proteomics into clinical applications.

8) References

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*ProtiFi and Covaris technologies are patented and patent-pending.