# Lossless reproducible sample preparation for simultaneous metabolomics and proteomics with universal S-Trap™ sample processing

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## 1) Introduction and background

- Proteomics, metabolomics and lipidomics provide mutually complementary data about the machinery of life and its chemical outputs. Ideally, these data are all acquired from exactly the same sample, both saving sample and ensuring sample homogeneity between different analyses.
- Many sample preparation techniques purify one class of molecule at the expense of others: e.g. “crash plates” for metabolomics or precipitations to isolate proteins from small molecules. With multiple omics analyses, the use of such common approaches require additional sample and sample prep time.
- Growing interest exists for a “one-pot” preparation or simultaneous extraction of proteins and small molecules for integrated analysis by proteomics and small molecule metabolomics/lipidomics. The majority of protocols use methanol/chloroform[1-5] with other protocols employing different organic solvents such as MTBE[6].
- Current “one-pot” workflows (and all protocols of references 1 – 6) make extensive use of centrifugation and handling of supernatants and/or phase layers, and protein pellets. These steps can be error prone (e.g. pellet loss; irreproducible recovery of different phases) and are difficult to automate.
- Here we present the use of S-Trap™ technology[7]* to reproducibly obtain both small molecule and peptide fractions from a single sample in a single system.

## 2) Lossless S-Trap™ sample processing

The Suspension-Trapping™ or S-Trap™[7] method is based on SDS-mediated protein solubilization (SDS ≤ 15%) and subsequent protein capture in the submicron pores of the S-Trap™. There, proteins are presented with extremely high surface area to volume, cleaned of detergent and contaminants and digested in-trap with proteases. In this work, we separate proteins from small molecules by acetonitrile precipitation atop the protein trap (other organics may also be used) and fully recover and process precipitated protein using 5% SDS. Thus, a single workflow using a single consumable affords samples for both proteomics and metabolomics/lipidomics from exactly the same sample, minimizing losses and maximizing reproducibility and automatability.

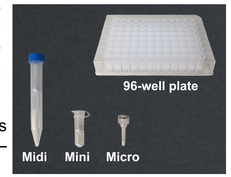


**Fig. 1:** Available formats of S-Traps™. Micros handle < 100 µg, minis and the 96-well plate 100 – 300 µg and midis > 300 µg.

## 3) Steps of lossless S-Trap™ sample processing

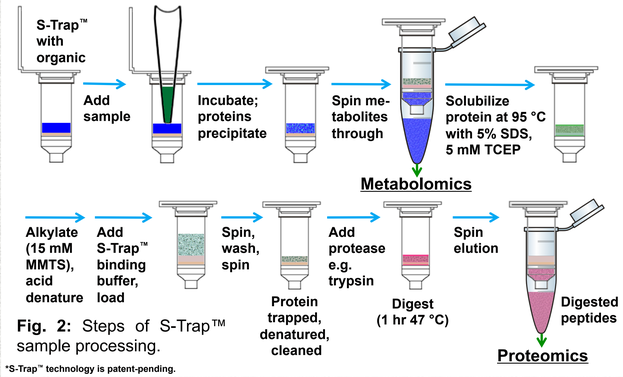


**Fig. 2:** Steps of S-Trap™ sample processing.

*S-Trap™ technology is patent-pending.

## 4) Materials and methods

96-well plate and mini S-Trap™ units were obtained from ProtiFi LLC (www.protifi.com, Huntington, NY). Pooled human plasma from 300 males and 300 females was obtained from Golden West Diagnostics (Temecula, California). Acetone powders from rabbit muscle, thymus and brain were purchased from Pel-Freez Biologicals (Rogers, AR). Chemicals and solvents were reagent (or better) or HPLC grade, respectively. 150 uL of acetonitrile cooled to -20 C was added to S-Trap™ mini units on ice and 1 uL biological sample (e.g. plasma) was added to the organic solvent and mixed. After incubation on ice for 15 min, metabolites were collected by centrifugation and dried down. 125 uL of 5% SDS in 50 mM TEAB containing 5 mM TCEP was added to the S-Traps, centrifuged into the trap and protein was dissolved for 10 min at 95 C. Traps were rinsed once with 50 uL water and alkylated by the addition of MMTS to 15 mM. The standard S-Trap procedure was subsequently followed: acidification, binding buffer addition, column binding, washing and digestion with trypsin as per standard protocols. A minimum of 5 – 6 sample replicates were independently prepared from the same pool. Peptides were analyzed on an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific), equipped with a nano-ion spray source was coupled to an EASY-nLC 1200 system (Thermo Scientific, 75-µm analytical column with an 8-µm emitter packed to 25cm with ReproSil-Pur C18-AQ, 1.9 um) using an 84 min gradient. Eluted peptides were directly electrosprayed into the Orbitrap Fusion Lumos mass spectrometer with the application of a distal 2.3 kV spray voltage and a capillary temperature of 300°C. Full-scan mass spectrum (Res=60,000; 400-1600 m/z) were followed by MS/MS using the “Top Speed” method for selection. High-energy collisional dissociation (HCD) was used with the normalized collision energy set to 35 for fragmentation, the isolation width set to 1.2 and a duration of 10 seconds was set for the dynamic exclusion with an exclusion mass width of 10 ppm. Metabolite fractions were analyzed on an Orbitrap QE in 20 min runs using both positive and negative ionization modes. Compound Discoverer and Proteome Discoverer (Thermo) were used to search and quantify analytes. Reproducibility assessments were done in Excel.

## 5) Experimental design

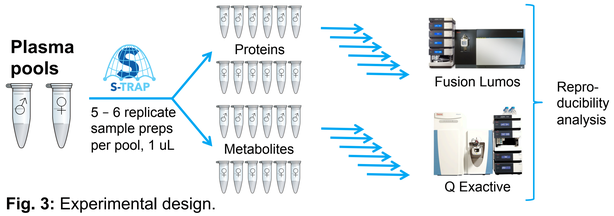


**Fig. 3:** Experimental design.

## 6) SDS-mediated protein solubilization

The majority of variation in sample preparation is often due to variable analyte recovery. S-Trap™ processing uses high concentrations of SDS (typically ~5% - 8%) to reproducibly solubilize and handle protein samples. This concentration is highly effective at recovering even poorly soluble proteins such as proteins precipitated from organics, membrane proteins or fibrous materials like chromatin, muscle, etc. Under these conditions, proteases and phosphatases are inactivated, reducing or eliminating the need for inhibitors and thus reducing cost.

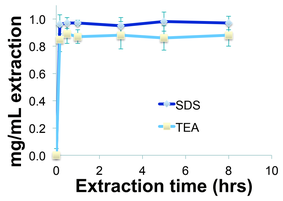


**Fig. 4:** Dried blood spots (Noviplex™ Plasma Prep Cards with the red blood cell fraction removed) were extracted with triethylammonium bicarbonate (pH 7.4, 50 mM) containing or lacking 5% SDS. Extraction kinetics were rapid. SDS consistently extracted more.

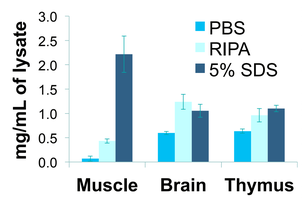


**Fig. 5:** SDS-mediated solubilization of poorly soluble proteins. Dry acetone powders were weighed in triplicate. Various lysis buffers were added, samples sonicated, incubated on an end-over-end rotator and assayed for protein concentration.

## 7) Reproducibility of proteomics sample preps

Replicates of proteomics sample preparations by S-Trap™ were highly reproducible. Among 347 proteins identified and quantified in all plasma runs, the median CV was consistently < 10%. 54.3% of quantifications had CVs < 10% and 68.2% had CVs < 15%. These results can be improved by the use of DIA and/or technical replicates (1 technical replicate/prep here).

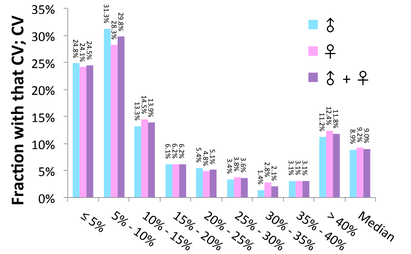


**Fig. 6**: Histogram of CVs for proteins quantified from males, females, and males and females combined. Rightmost median values are CVs, all other values are fraction of total quantifications.

## 8) Reproducibility of metabolomics sample preps

Replicates of metabolomics sample preparations by S-Trap™ were highly reproducible. 76 and 118 metabolites were identified and quantified in positive and negative ionization, respectively. Median CVs remained < 10%. 52.4% of quantifications had CVs < 10% and 65.7% had CVs < 15%. These results can be improved by the use of technical replicates (1 technical replicate/prep here).

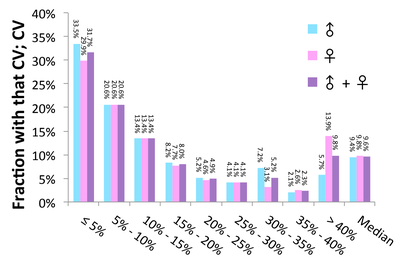


**Fig. 7**: Histogram of CVs for metabolites quantified from males, females, and males and females combined. Rightmost median values are CVs, all other values are fraction of total quantifications.

## 9) Overall reproducibility

Taken together, S-Trap™ sample processing was highly reproducible for both the preparation of small molecule and peptide fractions. For both datasets taken as a whole, all median CVs remained < 10%. 53.5% of quantifications had CVs < 10% and 67.2% had CVs < 15%. The variability of proteomics and metabolomics quantifications is notably similar, despite being obtained on two different systems.

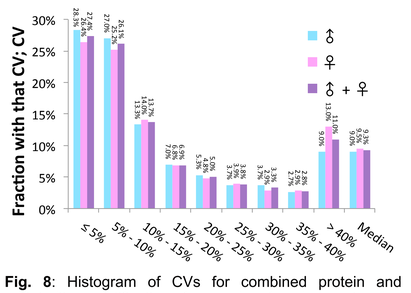


**Fig. 8**: Histogram of CVs for combined protein and metabolite quantifications from males, females, and males and females combined. Rightmost median values are CVs, all other values are fraction of total quantifications.

## 10) Variability as a function of observed counts

As a molecular “bean counter,” the reliability of mass spectrometric measurements will always be a function of the intensity of observation i.e. number of counts i.e. the depth of sampling. The variability of quantifications for both peptides and small molecules showed a marked dependence on the average total number of counts. Replicate and/or targeted analysis to increase counts of observation can improve measurement certainty.

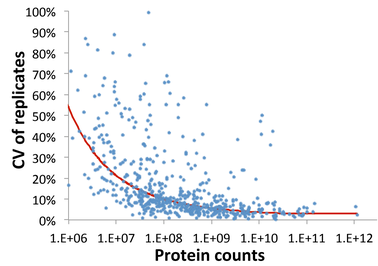


**Fig. 9**: CVs of protein quantifications as a function of intensity of observation. Trend-line in red.

## 11) Conclusion and future work

- S-Trap™ sample processing allows for reproducible sample preparation for both proteomics and metabolomics from the same sample; the use of a single sample reduces sample requirements and increases comparability of proteomics and metabolomics data.
- Sample processing time was increased by ~25 min over proteomics alone, extended by the incubation with organic and solubilization of precipitated protein.
- This same approach may be combined with biphasic systems such as chloroform, methanol and water, rather than the monophasic acetonitrile extraction demonstrated here. Specific lipid preparations are thus possible (cf. e.g. reference 6).
- The availability of S-Traps™ in a 96-well plate presents a direct route to high-throughput analysis of small molecules and proteins using one sample preparation. We anticipate this will be of use to facilitate “industrial scale” omics analyses, such as will be needed to effect personalized medicine.
- A full analysis of sources of variability of data, including distinction of technical machine variability from sample preparation variability, is underway.
- Studies are ongoing to determine the feasibility of processing precipitated protein with reduced solubilization.

## 12) References

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