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Suspension Trapping (S-Trap) Is Compatible with Typical Protein Extraction Buffers and Detergents for Bottom-Up Proteomics

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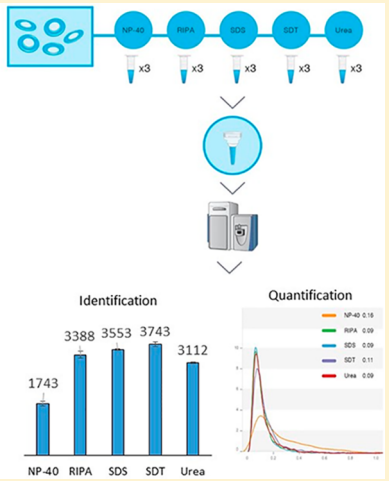
ABSTRACT: The analysis of cells and tissue by bottom-up proteomics starts with lysis, followed by in-solution digestion. Lysis buffers commonly used include detergents and other reagents for achieving efficient protein solubility. However, these reagents are, for the most part, incompatible with downstream analytical instrumentation. One method for in-solution digestion and cleanup, termed suspension trapping (S-Trap), has been recently introduced. We present an evaluation of the compatibility of commonly used lysis buffers with S-Trap: SDS, urea, NP-40, RIPA, and SDS with DTT (SDT). We show that S-Trap is compatible with all of the tested buffers, with SDS and SDT performing the best. On the basis of these data, we anticipate that the method will transform experimental planning for mass-spectrometry-based proteomics, making it far more flexible and tolerable of various lysis buffers. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD011665.



KEYWORDS: *proteomics, S-Trap, lysis, detergents, phosphoproteomics, mass spectrometry, sample preparation, label-free, SDS, SDT, urea, RIPA*

INTRODUCTION

In-solution digestion is commonly used in bottom-up proteomics, particularly for the analysis of whole-cell or tissue lysates. The process includes the extraction of proteins from the cells or tissue, followed by in-solution digestion. Efficient lysis of cells and tissue requires chaotropic agents such as urea, guanidine, or sodium dodecyl sulfate (SDS).[1,2] Detergents, such as NP-40, or a combination of several detergents are also used for protein extraction when milder conditions are needed.[3−5] All of these reagents must be removed from the sample prior to LC−MS analysis due to their incompatibility with the analytical instrumentation. They form peak clusters that mask all other signals in the mass spectrometer, and they can form salt crystals during electrospray ionization or clog the LC columns.

Numerous approaches are used to clean the samples, either before digestion, such as protein precipitation[6,7] and molecular-weight cutoff columns,[5] or postdigestion, such as C18 cartridges[8] or columns for detergent removal.[9] Each method has its advantages and disadvantages. Up until recently, there was no single, simple, and reproducible method that was compatible with the main lysis buffers used in mass-spectrometry-based proteomics.

Recently, a new method was introduced, called suspension trapping.[10−12] In a recent report, it was found to be superior to the popular filter-aided sample preparation (FASP) method.[10]

We present an evaluation of the compatibility of S-Trap with common lysis buffers. We compared the lysis of HeLa cells using urea, NP-40, SDS, RIPA, and SDS with DTT (SDT), followed by in-solution digestion with the S-Trap method.

EXPERIMENTAL PROCEDURES

Sample Preparation

HeLa cells were grown in DMEM medium, harvested, and washed twice with PBS. Identical pellets of 10^6 cells were lysed with the following buffers: 8 M urea, 1% NP-40, 5% SDS in 50 mM Tris-HCl, 4% SDS and 0.1 M dithiothreitol (SDT), and radioimmunoprecipitation assay (RIPA) comprising 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS in 50 mM Tris pH 8. Lysates with SDS, SDT, RIPA, and NP-40 were incubated at 96 °C for 5 min, followed by six cycles of 30 s of sonication (Bioruptor Pico, Diagenode, USA). Lysates with urea were incubated for 10 min at room temperature, followed by sonication.

Protein concentration was measured as follows: for samples containing SDS and NP-40, BCA assay (Thermo Scientific, USA); for samples containing urea, the Pierce 660 assay (Thermo Scientific, USA); and for the SDT (SDS+DTT), the Pierce 660 assay with IDCR (Thermo Scientific, USA). 50 μg

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of total protein was reduced with 5 mM dithiothreitol and alkylated with 10 mM iodoacetamide in the dark. Each sample was loaded onto S-Trap microcolumns (Protifi, USA) according to the manufacturer's instructions. In brief, after loading, samples were washed with 90:10% methanol/50 mM ammonium bicarbonate. Samples were then digested with trypsin (1:50 trypsin/protein) for 1.5 h at 47 °C. The digested peptides were eluted using 50 mM ammonium bicarbonate; trypsin was added to this fraction and incubated overnight at 37 °C. Two more elutions were made using 0.2% formic acid and 0.2% formic acid in 50% acetonitrile. The three elutions were pooled together and vacuum-centrifuged to dry. Samples were kept at −80 °C until analysis.

Immobilized Metal Affinity Chromatography

After the analysis of the 15 samples (five lysis buffers in triplicate), the replicates were pooled, and ∼150 μg of each sample was subjected to phosphopeptide enrichment. It was performed on a Bravo robot (Agilent Technologies) using AssayMAP Fe(III)-NTA, 5 μL cartridges (Agilent Technologies), according to the manufacturer's instructions. In brief, cartridges were primed and equilibrated with 50 μL of buffer A (99.9% ACN/0.1% TFA) and 100 μL if buffer C (80% ACN/19.9% H_2O/0.1% TFA), followed by sample loading in 100 μL of buffer C at 5 μL/min. Phosphopeptides were eluted with 120 μL of buffer B (99% H_2O/1% NH_3) at 5 μL/min. Three μL of formic acid was added to each sample for acidification. Prior to LC−MS analysis, all samples were dried down to the volume of 15 μL.

Liquid Chromatography−Mass Spectrometry

ULC−MS-grade solvents were used for all chromatographic steps. Each sample was loaded using split-less nano-ultra performance liquid chromatography (10 kpsi nanoAcquity; Waters, USA). The mobile phase was: (A) H_2O + 0.1% formic acid and (B) acetonitrile +0.1% formic acid. Desalting of the samples was performed online using a reversed-phase Symmetry C18 trapping column (180 μm internal diameter, 20 mm length, 5 μm particle size; Waters). The peptides were then separated using an HSS T3 nanocolumn (75 μm internal diameter, 250 mm length, 1.8 μm particle size; Waters) at 0.35 μL/min. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4 to 20% B in 155 min, 20 to 90% B in 5 min, maintained at 90% for 5 min, and then back to initial conditions.

The nanoUPLC was coupled online through a nanoESI emitter (10 μm tip; New Objective; Woburn, MA) to a quadrupole Orbitrap mass spectrometer (Q Exactive HF, Thermo Scientific, USA) using a FlexIon nanospray apparatus (Thermo Scientific).

Data were acquired in data-dependent acquisition (DDA) mode using a top10 method. MS1 resolution was set to 120 000 (at 200 m/z), mass range was 375−1650m/z, AGC was 3e6, and maximum injection time was set to 60 ms. MS2 resolution was set to 15 000, quadrupole isolation was 1.7 m/z, AGC was 1e5, dynamic exclusion was 45 s, and maximum injection time was 60 ms. Only charge states 2 to 8 were allowed for MS/MS triggering.

Data Processing

Raw data were analyzed using the MaxQuant software suite 1.6.0.16 (www.maxquant.org) with the Andromeda search engine.[13] The higher-energy collisional dissociation (HCD) MS/MS spectra were searched against an in silico tryptic digest of *Homo sapiens* proteins from the UniProt/Swiss-Prot sequence database (v. March 2018) containing 20 547 sequences, including common contaminant proteins. All MS/MS spectra were searched with the following MaxQuant parameters: acetyl (protein N-terminus) and methionine oxidation as variable modifications; cysteine carbamidomethylation was set as fixed modification for all samples, except for the SDT samples in which case carbamidomethylation was set as variable modification; max 2 missed cleavages; and precursors were initially matched to 4.5 ppm tolerance and 20 ppm for fragment spectra. Peptide spectrum matches and proteins were automatically filtered to a 1% false discovery rate based on Andromeda score, peptide length, and individual peptide mass errors. Processing was conducted without a match between runs.

Proteins were identified and quantified based on at least two unique peptides and based on the label-free quantification (LFQ) values reported by MaxQuant.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository[14] with the data set identifier PXD011665.

RESULTS

We compared five lysis buffers for compatibility with the suspension trapping (S-Trap) method of in-solution digestion. HeLa cells were lysed with urea, NP-40, SDS, SDS with dithiothreitol (SDT), and radioimmunoprecipitation assay (RIPA) in triplicate (Figure 1). The performance was evaluated based on the following criteria: proteomic coverage, identification reproducibility, digestion efficiency, and quantitative reproducibility.

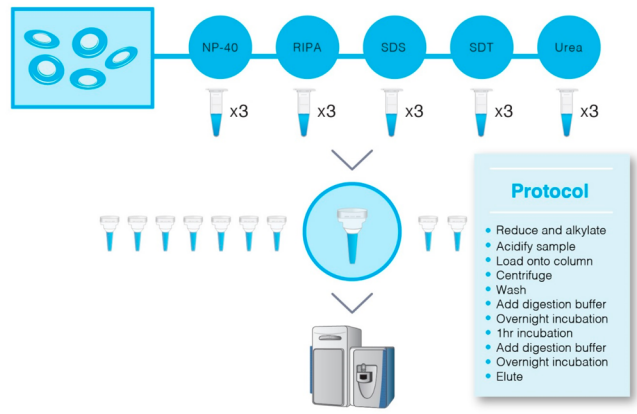

Figure 1. Outline of the experimental design. HeLa cell pellets were aliquoted to 1×10^6 cells each and lysed with 1% NP-40, 5% SDS, 5% SDS with DTT, RIPA, and 8 M urea in triplicate. 50 μg of total protein was loaded onto S-Trap microcolumns and subjected to LC−MS/MS analysis.

Proteomic Coverage

Our evaluation started with comparing the number of proteins and peptides we could identify with the S-Trap in the different lysis buffers. Figure 2A,B shows that with the exception of NP-40, with all buffers, over 3000 proteins were identified (based on at least two unique peptides). The best performing lysis buffer was the SDT, with an average of 3743 proteins. However, because of the presence of a high concentration of DTT, only 43% of cysteines were alkylated. For this type of

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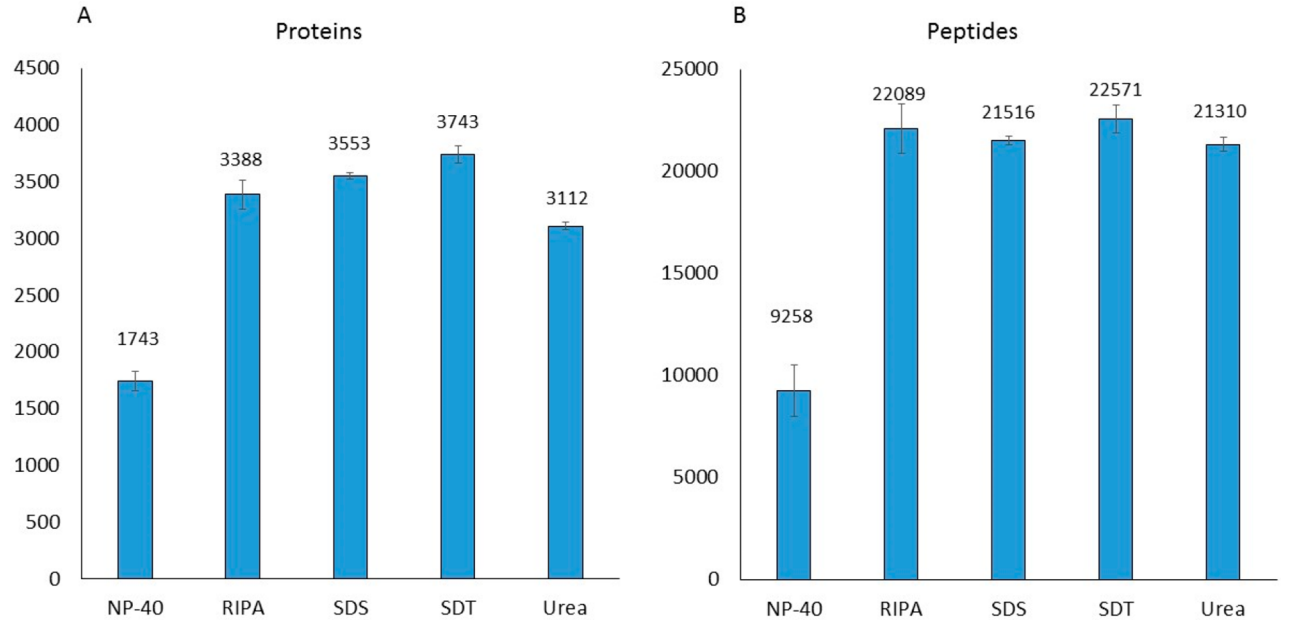

Figure 2. (A) Mean number of proteins identified (based on two or more unique peptides) with each lysis buffer. (B) Mean number of peptides identified using each lysis buffer. Error bars represent standard deviation.

lysis buffer, it may be worth alkylating on the S-Trap column after washing with the lysis buffer. In this experiment, this procedure was not tested.

The lysis using NP-40 resulted in only 1743 identified proteins. This is not surprising because NP-40 is a mild detergent and on its own is not enough to solubilize all proteins in the cell. Figure 3 shows the overlap in identified proteins. NP-40 presented with the most variability in terms of replicating protein identification.

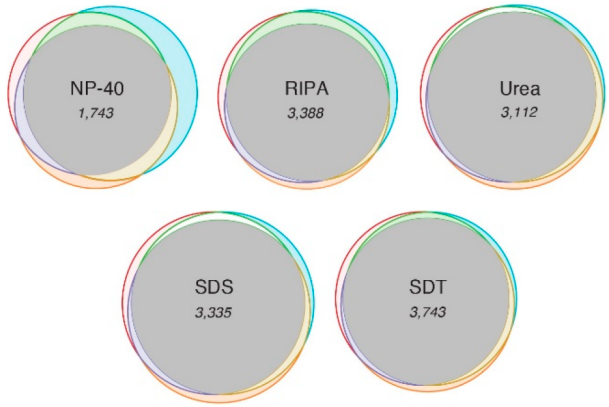

Figure 3. Venn diagrams of the overlap in protein identification (minimum two unique peptides per protein) across the replicates for each lysis buffer.

Digestion Efficiency

One of the most important factors for evaluating new methods, particularly if the focus is on quantification, is the digestion efficiency. Figure 4 shows bar graphs of the percentage of missed cleavages in each replicate. It can be seen that in all samples, >75% of the peptides were fully cleaved. The SDT buffer performed slightly worse than others, showing a slightly higher rate of missed cleavage peptides.

Quantitative Reproducibility

Proteins were quantified using MS1-based LFQ, as reported by the MaxQuant software. For each protein, a coefficient of variation was calculated based on the LFQ intensity in each replicate of each lysis buffer. Figure 5 shows a density plot (normalized histogram) of the quantitative coefficient of variation for each lysis buffer. Apart from NP-40, >80% of the proteins were quantified with a coefficient of variation <20%, showing that the S-Trap is highly reproducible not only in the identification but also in the relative quantification of the proteins.

Phosphoproteomics

Lastly, we took the leftover samples that were used in the aforementioned analysis, pooled the replicates, and subjected them to phosphopeptide enrichment. Each sample contained up to 150 μg of total peptides. We used Fe(III) immobilized metal affinity chromatography (IMAC) using the AssayMAP tips on a robotic system. The results of this analysis are shown in Figure 6. The best performing buffer was SDT, with 7815 phosphopeptides. This shows the compatibility of the S-Trap not only for protein expression but also for phosphopeptide analysis.

CONCLUSIONS

Cell lysis is the first step of sample preparation for mass-spectrometry-based proteomic analysis. Over the years, many lysis methods have been reported for cells and tissue. All of them combine the use of detergents or chaotropic agents or a combination of both. In all cases, the sample needs to be cleaned of these reagents prior to LC−MS analysis, regardless of whether it is done before digestion or after. This is due to the incompatibility of detergents and chaotropic reagents with LC−MS, as they might clog the LC column, cause high background of ions, or soil the mass spectrometer, leading to the loss of performance.

We presented an evaluation of a new method for in-solution digestion and cleanup of biological samples for bottom-up

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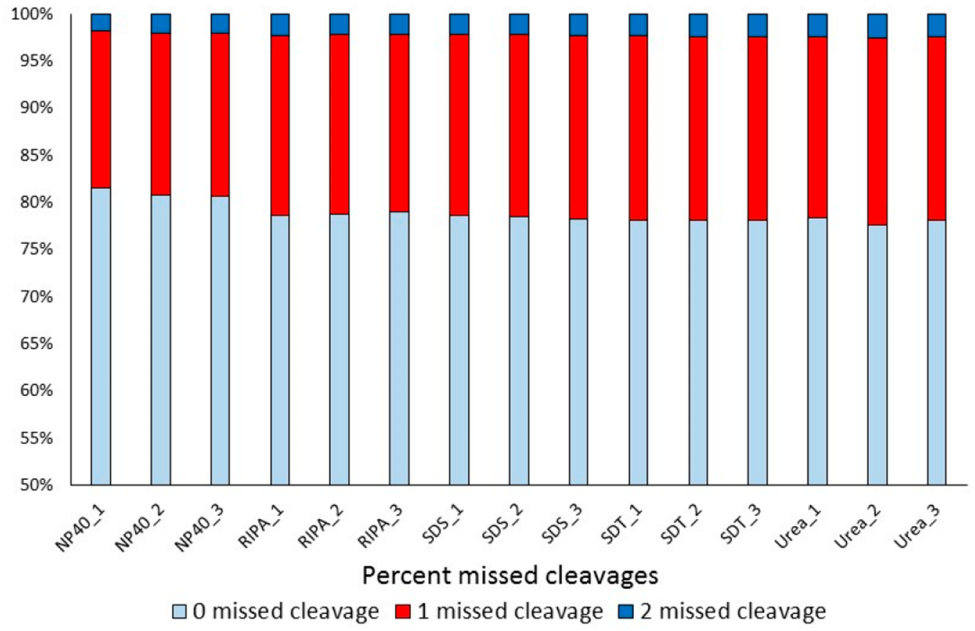

Figure 4. Comparison of the missed cleavages in each replicate.

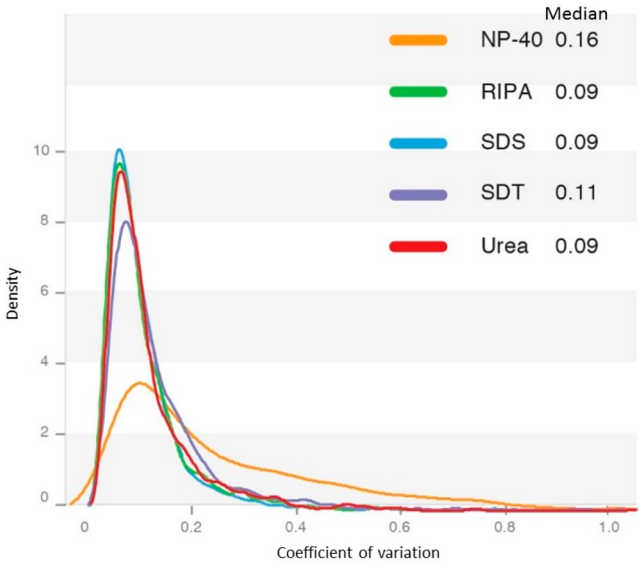

Figure 5. Density plots of the LFQ intensity coefficient of variation (CV) across the replicates of each lysis buffer. 80% of the proteins each quantified with CV <0.2.

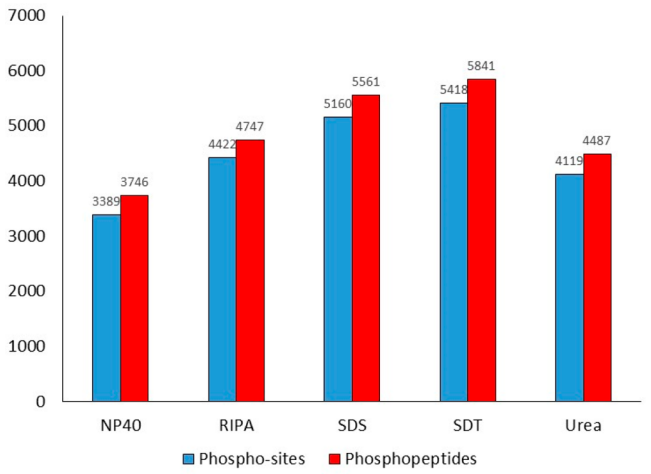

Figure 6. Phosphopeptides and phosphosites detected in each sample. In this case, replicates were pooled prior to enrichment to reach 150 μg total protein per sample.

proteomics. We showed that the method produces efficient digestion, leads to high proteomic coverage, and is highly reproducible. We also showed that the resulting peptide mixtures are compatible with phosphopeptide enrichment. The presented protocol is straightforward and does not require desalting after digestion. On the basis of these results, we conclude that the optimal lysis buffer is the SDS.

The fact that S-Trap is compatible with most detergents means that the proteomics laboratory does not need to worry about the composition of the buffer prior to digestion. Because proteins are trapped so efficiently on the S-Trap column, the sample can be efficiently washed of any nonprotein contaminant. This method has already transformed our experimental planning and execution, and we anticipate that it will do the same for other laboratories.

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Notes

The authors declare no competing financial interest.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD011665.

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