Sample Preparation for Mass Spectrometry-Based Proteomics; from Proteomes to Peptides

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Abstract

Mass spectrometry (MS) has become the predominant technology to analyze proteins due to it ability to identify and characterize proteins and their modifications with high sensitivity and selectivity (Aebersold and Mann, Nature 422(6928):198–207, 2003; Han et al., Curr Opin Chem Biol 12(5):483–490, 2008). While mass spectrometry instruments have improved rapidly over the past couple of decades, mass spectrometry results have remained largely dependent on sample preparation and quality. Sample ionization and mass measurements are susceptible to a wide variety of interferences, including buffers, salts, polymers, and detergents. These contaminants also impair MS system performance, often requiring time consuming maintenance or costly repairs to restore function. The goal of this chapter is to describe the rationale, considerations, and general techniques used to prepare samples for proteomic mass spectrometry analysis.

Keywords

Protein chromatography • Protein extraction • Lysis • Protein depletion or enrichment • Digestion • In-gel digestion • In-solution digestion • Filter-assisted sample preparation (FASP) • Digestion comparison

3.1 Overview

Due to the complexity of proteomic samples and the wide variety of sample preparation techniques, a proteomics researcher must first determine the right experimental strategy. A successful proteomics experiment requires the integration of good sample preparation, instrumentation, and software (Fig. 3.1). Therefore, it is important to understand the goals and expectations of the project and to choose and optimize the best sample preparation method accordingly. For example, the sample preparation requirements for protein identification from

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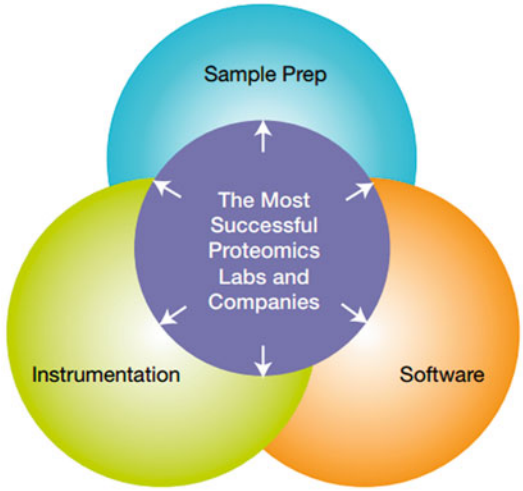

Fig. 3.1 The key to proteomics success. Successful proteomics laboratories and companies recognize the importance of sophisticated sample preparation, instrumentation, and software technologies and skills. Workflows designed to maximize the overlap between these complementary technologies are an effective means of improving proteomics research

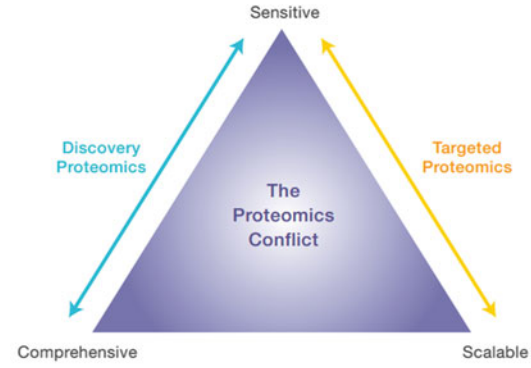

Fig. 3.2 The proteomics conflict. It is impossible to optimize sensitivity, throughput and comprehensiveness simultaneously. Discovery proteomics strategies optimize sensitivity and comprehensiveness with few samples. Targeted proteomics strategies optimize sensitivity and scalability by limiting the number of monitored features. Note that comprehensive analysis with reasonable throughput is enabled by sample multiplexing with mass tag reagents

a gel slice are very different from the requirements to identify protein interaction networks, measure changes in the mitochondrial proteome, understand protein phosphorylation and signaling in cancer, or identify protein biomarkers of cancer metastasis in plasma [3–6]. Unlike genomic or transcriptomic research, there is no "standard" universal sample preparation method for proteomics.

Additionally, proteomics experiments must balance the competing needs for sensitive and complete proteome coverage with the scalability of analyses (Fig. 3.2). Proteomic strategies to improve proteome coverage require multidimensional fractionation; however, this fractionation increases the sample analysis time and sacrifices throughput [7, 8]. Alternatively, MS acquisition strategies that improve the sensitivity, reproducibility, and throughput of protein quantification, such as selected reaction monitoring (SRM) or parallel reaction monitoring (PRM), limit the number of features that can be monitored [9, 10]. For this reason, proteomics research is generally divided into three categories: protein identification and characterization, proteome profiling, and targeted protein analysis.

Protein identification and characterization is commonly performed to identify protein isoforms, splice variants, post-translational modifications, and interacting proteins [11]. These studies are typically performed after protein separation using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and may also involve a protein enrichment step, such as immunoprecipitation. In contrast, proteomic profiling is typically performed on whole protein or sub-proteome extracts digested in solution. This comprehensive approach requires more instrument analysis time per sample to maximize the number of protein identifications at the expense of the number of samples that can be analyzed. Isobaric mass tags (e.g. iTRAQ and TMT) can help to address this sample throughput limitation by allowing multiple samples to be combined into a single LC-MS analysis [12–14]. Targeted protein analysis limits the number of features that are monitored to a pre-selected list of target peptides and their transitions. These methods optimize sample preparation, chromatography, instrument tuning, and fragmentation to achieve the highest sensitivity and throughput for

hundreds of samples. Ultimately, a sample preparation strategy should be chosen which generates the most biologically relevant or useful data possible for a given experiment.

Protein analysis using tandem mass spectrometry (MS/MS, or MS^n) can be performed on intact proteins ("top-down" proteomics) or protein digests ("bottom-up" proteomics). Top-down proteomics is a growing field, as it permits nearly complete protein sequence coverage and enables simultaneous characterization of protein isoforms and modifications [15, 16]. However, top-down analysis is currently limited to proteins less than ~50,000 Da and requires high resolution MS instrumentation (>100,000 resolving power) to accurately identify proteins and protein isoforms. Recently, "middle-down" strategies have also been developed to reduce the sizes of intact proteins through partial digestion or using proteases that cleave at rare sites or at specific positions within a protein (e.g. antibodies, [17, 18]). Sample preparation for intact proteins typically involves multi-dimensional protein fractionation to reduce sample complexity and protein desalting to remove residual salts or other impurities that may form adducts during ionization.

Bottom-up proteomic strategies represent the vast majority of MS proteomic analyses. These methods use proteases to digest proteins at specific amino acids into peptides with a predictable terminus. Unlike proteins, peptides are more easily separated by reverse phase HPLC and ionize well by electrospray or matrix-assisted laser desorption ionization (MALDI). Importantly, peptides fragment during MS/MS to yield amino acid sequence information. Similar to proteins, multi-dimensional fractionation of peptides can be used to reduce sample complexity [19] but removal of salts, detergents and other impurities can be more difficult at the peptide level than the protein level. As peptide fractionation, liquid chromatography (LC), and MS analysis are addressed in other chapters, this chapter will primarily focus on bottom-up protein sample preparation strategies prior to LC-MS/MS analysis.

The quality and consistency of sample preparation influences the time and cost of MS analysis and the reliability of the results. For MS-based proteomics to reach its full potential as a routinely used detection technology in research and clinical settings, variability associated with the sample preparation steps that precede MS analysis must be addressed. Despite extensive literature describing various MS sample preparation methods explained below and elsewhere, there is little standardization among methods. This results in confusion for those new to MS sample preparation techniques and high variability in MS analysis results, even among expert MS laboratories.

3.2 Protein Extraction

Tissue or cell lysis is the first step in protein extraction and solubilization. Numerous techniques have been developed to obtain the highest protein yield for different organisms, sample types, subcellular fractions, or specific proteins. Due to the diversity of tissue and cell types, both physical disruption and reagent-based methods are often required to extract cellular proteins. Physical lysis equipment, such as homogenizers, bead beaters, and sonicators, are commonly used to disrupt tissues or cells in order to extract cellular contents and shear DNA. In contrast, reagent-based methods use denaturants or detergents to lyse cells and solubilize proteins. Cell lysis also liberates proteases and other catabolic enzymes so broad-spectrum protease and phosphatase inhibitor cocktails are typically included during sample preparation to prevent nonspecific proteolysis and loss of protein phosphorylation, respectively.

Through the use of different buffers, detergents and salts, cell lysis protocols can be optimized for the best protein extraction for a particular sample or protein fraction. Strong denaturants (e.g. urea or guanidine) and ionic detergents (e.g. sodium dodecyl sulfate (SDS) or deoxycholate (SDC)) solubilize membrane proteins and denature proteins. Non-ionic or

zwitterionic detergents (e.g. Triton X-100, NP-40, digitonin, or CHAPS) have a lower critical micelle concentration and require lower detergent concentrations to solubilize proteins [20, 21]. These detergents generally solubilize membrane proteins and protein complexes with less denaturation and disruption of protein-protein interactions [21].

Unfortunately, many detergents used to solubilize proteins cause significant problems during downstream mass spectrometry analysis if they are not completely removed. In addition to cell lysis buffers, detergents used to clean laboratory glassware may also contaminate samples and LC solvents. Detergents present in the sample can:

1. Contaminate and foul autosampler needles, valves, connectors, and lines
2. Affect liquid chromatography by reducing column capacity and performance
3. Affect crystallization prior to matrix assisted laser desorption ionization (MALDI) sample analysis;
4. Suppress electrospray ionization (ESI) prior to introduction into the mass spectrometer
5. Deposit in the mass spectrometer, interfering with the spectra and reducing sensitivity of the instrument.

Flexible tubing or poor quality plastic consumables can also leach phthalates and other contaminants that can interfere with downstream LC-MS analysis [22]. Both phthalates and detergents ionize very well and overwhelm peptide signals. Polydisperse detergents, such as Triton X-100, Tween or NP-40, contain a distribution of variable length polyethylene glycol (PEG) chains that often elute throughout the LC gradient as a family of peaks separated by 44 Da mass units and overwhelm the LC-MS results. Fortunately, these leachables and detergents can often be removed by gel electrophoresis, protein precipitation, or filter-assisted sample preparation (FASP) techniques described later in this chapter.

While all detergents can affect downstream LC-MS analysis, N-octyl-beta-glucoside and octylthioglucoside are considered more compatible with mass spectrometry because they are dialyzable and monodisperse (i.e. homogeneous) [23]. In addition, a variety of mass spectrometry-compatible detergents are commercially available. Invitrosol (Thermo Scientific) contains several monodisperse detergents that elute in regions of the HPLC gradient that do not interfere with peptides or their chromatography. Cleavable detergents, such as ProteaseMax (Promega), Rapigest (Waters), PPS Silent Surfactant (Expedeon), or Progenta (Protea), degrade with heat or at low pH into products that do not interfere with LC-MS. As digestion requires incubation at 37 °C and LC-MS loading buffers contain formic acid or trifluoroacetic acid, sample preparation workflows do not require any significant modification to use these MS-compatible detergents [24].

3.3 Protein Depletion or Enrichment

Depending on the protein source and the copy number per cell, there can be a tremendous difference in the concentration between the lowest and most abundant proteins. For mammalian tissues and cell lines, protein expression can range over 6–9 orders of magnitude. For serum and plasma samples, the dynamic range can be greater than 12 orders of magnitude with serum albumin representing over 50 % of the protein content [25]. In order to get an adequate depth of protein coverage in serum, to identify relevant biomarkers, abundant protein depletion is required. Although affinity chromatography using Cibacron blue dye can be used to remove albumin, immunoaffinity using antibodies is typically required to remove other abundant proteins such as immunoglobulins, transferrin, fibrinogen, and apo-lipoproteins [26]. One advantage of using antibodies for immunodepletion is that one sample preparation technique can be used to remove the top 2–20 most abundant proteins depending on the product used. Another is that the depletion resins can be regenerated for multiple uses; though this can affect protein depletion reproducibility over time.

Protein enrichment techniques are commonly overlooked during protein sample preparation but may be necessary in order to identify and quantify biologically relevant proteins which are typically in lower abundance. One method of protein enrichment is subcellular fractionation, which separates proteins by location in a particular cellular compartment or organelle. Subcellular fractionation using sucrose density gradient centrifugation can separate vesicles and organelles including the nucleus, mitochondria, or chloroplasts from cytosolic and vesicle proteins [27, 28]. Differential extraction is another subcellular fractionation technique which uses detergents to selectively solubilize nuclear, chromatin-bound, membrane, cytosolic, and cytoskeletal proteins [29]. Another method of protein enrichment is through protein modifications. Cell surface proteins which are glycosylated can be enriched by chemical labeling of oxidized glycans, metabolic incorporation of azide-containing sugars [30–32], or lectin affinity [33]. Phosphoproteins can be enriched with immobilized metal affinity chromatography [34]. Activity-based chemical probes are another method for enrichment of enzyme subclasses such as kinases, hydrolases, and oxidases [35, 36]. Finally, affinity capture using immunoprecipitation is the method of choice for enrichment of specific protein targets or protein complexes as this technique provides the highest selectivity and sensitivity for the lowest abundant proteins [37].

3.4 Protein Preparation

Unfortunately, many protein extraction, fractionation, enrichment and depletion methods introduce salts, buffers, detergents, and other contaminants which are not MS compatible. Because of the relative difference in molecular weight, it is simplest and preferable to remove these small molecule contaminants before protein digestion. There are a variety of options to remove these small molecules, including gel electrophoresis, chromatography, dialysis, buffer exchange, size exclusion, and protein precipitation [38, 39]. Gel electrophoresis is an inexpensive, straightforward method for the removal of salts, detergents, and other small molecules prior to in-gel digestion. However, keratins from skin and dust are common contaminants which can be introduced when pouring and handling gels so it is imperative to always wear gloves and to use MS grade reagents to minimize this contamination.

Reverse phase C4 or C8 cartridges can remove salts from proteins but concentrate non-ionic detergents and may have poor recovery of hydrophilic proteins. Strong cation exchange resins can remove anionic detergents, like deoxycholate or sodium dodecyl sulfate (SDS), but typically require salts for protein elution which then have to be removed before LC-MS analysis. Dialysis membranes and cassettes are available with a variety of molecular weight cut-offs (MWCO) and can effectively exchange buffer components to remove contaminants; but dialysis is relatively slow, requires multiple buffer changes, and may be difficult with small volumes. Spin columns or stirred-cell pressure devices with MWCO membranes can rapidly exchange buffers to remove small molecule contaminants and concentrate samples. These MWCO devices allow sequential buffer exchange steps to be performed and can be used for complete MS sample preparation in the filter-assisted sample preparation (FASP) methods. Size exclusion resins retain small molecules in porous beads while excluding proteins enabling rapid and efficient buffer exchange with minimal sample loss, especially in a spin column format. Notably, of all of the desalting methods available, precipitation with organic solvents such as acetone or methanol/chloroform with or without organic acids (e.g. TCA or TFA) is the most common method for desalting proteins prior to MS sample preparation as it the least expensive, simplest and most scalable option.

3.5 Protein Digestion

Trypsin is the most commonly used protease for MS sample preparation because of its high

activity, selectivity and relatively low cost. Trypsin cleaves proteins to generate peptides with a lysine or arginine residue at the carboxy terminus [40]. These basic amino acids at the end of every tryptic peptide improve peptide ionization and MS/MS fragmentation for peptide identification. Although trypsin is the most popular enzyme used for protein digestion, some protein sequences are not efficiently cleaved by trypsin or do not contain basic amino acids spaced close or far enough apart to generate peptides which can be used for protein identification. Trypsin digestion is less efficient at lysine and arginine residues followed by proline, repeated basic residues (e.g. KK, RK), or in the presence of post translational modifications (e.g. methylation, acetylation), resulting in missed cleavages [41]. Some tryptic peptides may be too small to retain on reversed phase LC columns or are not unique for a particular protein. Others may be too large and hydrophobic to identify by LC-MS. For example, 56 % of the tryptic peptides in yeast are $\leq$6 amino acids long, while 97 % of peptides identified by LC-MS are 7–35 amino acids [42]. These short or extremely long unidentified peptides result in incomplete protein sequence coverage, resulting in missing specific peptide sequences or sites of posttranslational modifications.

For more comprehensive proteome coverage, alternative proteases are often used to generate different peptide sequences that may not be identified from tryptic digests. Partial digestion with specific or non-selective proteases, like elastase or proteinase K, have been used to increase protein sequence coverage; but these proteases also increase the complexity and variability of digestion, making it more difficult to reproducibly identify the same peptides and proteins in replicate samples [43, 44]. Proteases with distinct cleavage specificities, such as ArgC, AspN, chymotrypsin, GluC, LysC, or LysN, produce complementary sequence information which can be combined to improve sequence coverage. This multi-enzyme approach has been used successfully by multiple laboratories to increase the number of protein identifications $>$10–15 % and improve the average sequence coverage by 60–160 % [42, 45–47]. Different proteases have also been shown to provide a unique repertoire of phosphopeptides which are not observed in tryptic digests [48]. Therefore, a multiple enzyme strategy is recommended for comprehensive analysis of single proteins or complex proteomes.

Multiple studies have demonstrated that chaotropes, solvents and detergents increase the efficiency of protein digestion [49, 50]. These reagents assist in the solubilization and unfolding of proteins, especially integral and transmembrane proteins or hydrophobic stretches of protein sequence. Efficient digestion is important to maximize the number of peptides and proteins identified in a sample, and complete digestion permits the reproducible quantitation of peptides. Organic solvent additives, such as 5–20 % acetonitrile (ACN), trifluoroethanol, and methanol have been shown to improve digestion efficiency and only require vacuum centrifugation or dilution to be compatible with LC-MS analysis. Urea and guanidine chaotropes also improve protein solubilization and digestion efficiency. These salts are easily removed from proteins by desalting on dialysis, or from peptides by using reverse phase C18 tips, cartridges, or trap columns. However, urea can modify lysine residues, resulting in carbamylation artifacts [51] and some proteases are not active in guanidine. Finally, some detergents which are used for protein extraction have also been shown to aid protein digestion. Depending on the detergent, these reagents can be removed after digestion by phase transfer, detergent removal resins, or hydrolysis with low pH [24, 50, 52]. Interestingly, it is reported that a combination of 1 M guanidine and 20 % ACN with any MS compatible detergent greatly improves the digestion efficiency and specificity over any one of these additives alone [24]. While the effects of solvents, chaotropes, and detergents have been well studied for trypsin digestion, and to a lesser extent for LysC digestion, the effects of these additives on other proteases are not well understood.

3.6 Peptide Preparation: In-Gel Digestion

Once the proteins in a complex sample are solubilized, there are three general approaches to prepare protein digests: in-gel digestion, in-solution digestion, and filter-assisted sample preparation (Fig. 3.3). All three of these methods remove contaminating detergents and other small molecules, reduce and alkylate proteins, digest proteins to peptides, and prepare peptides for mass spectrometry analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is the most common technique for protein analysis [39, 53]. Gel electrophoresis is a simple, inexpensive and a relatively high resolution protein separation method that can be employed in either one dimension (1D) to resolve proteins by molecular weight or two dimensions (2D) to resolve proteins by isoelectric point and molecular weight [54]. Although 2D PAGE is not compatible with salts and ionic detergents, 1D SDS-PAGE can easily remove these and other substances which may interfere with LC-MS analysis. In fact, many academic proteomic core labs prefer or require samples to be provided in gels or gel slices because this method is so effective for sample clean up. Depending on the depth of analysis, a single band can be excised or a complex sample can then be excised as a set of gel slices in a method often referred to as GeLC-MS [55]. Another advantage of gel-based fractionation methods is that they can reduce sample complexity and separate highly abundant proteins from lower abundant proteins. Since all of the peptides from the respective protein(s) are contained in a single gel band, spot or fraction, protein sequence coverage and posttranslational modification mapping is also improved.

After gel electrophoresis, separated proteins are detected and visualized with a variety of gel stains, including Coomassie Blue, Colloidal Coomassie, and glutaraldehyde-free silver stain. Gel bands containing protein(s) of interest are then excised, destained, reduced, and alkylated to improve digestion and peptide extraction [39]. Disulfide bonds prevent complete protein unfolding and limit proteolytic digestion. Peptides that remain linked by disulfides are also difficult to identify due to the complexity of the peptide fragment ion spectra. Protein disulfides are typically reduced with either dithiothreitol (DTT) or tris 2-carboxyethylphosphine (TCEP) in the presence of other denaturants (i.e. heat, SDS, urea, guanidine, etc.). Reduced cysteines are then alkylated with iodoacetamide, iodoacetic acid, chloroacetamide, 4-vinyl pyridine, or N-ethyl maleimide (NEM) to prevent oxidation [56–58]. Haloacetyl-containing alkylating agents are light sensitive and must be made fresh. Alkylation reactions should be performed at pH 8.0 to avoid alkylation at other amino acids, and excess reagent should be quenched with DTT to prevent side reactions and over-alkylation of proteins. After reduction and alkylation, gel bands are digested with a protease; and the peptides are extracted using standard techniques [39]. While in-gel digestion is more prone to incomplete or less reproducible digestion and lower recovery of peptides relative to in-solution option (50–70 % recovery), gel electrophoresis remains an important sample preparation technique prior to MS analysis (Fig. 3.3, and Supplement Method 1).

3.7 Peptide Preparation: In-Solution Digestion

In-solution digestion is a popular alternative to in-gel digestion, because it requires fewer steps and can be scaled for the analysis of samples containing less than 10 µg or greater than 1 mg of protein. For this method, proteins are first denatured with detergents and heat or with urea or guanidine chaotropes. Disulfide bonds between cysteine residues are reduced and alkylated and then sample contaminants are typically removed by precipitation prior to digestion and cleanup. As stated above, urea has been used for many years but is not recommended because it must be made fresh as the formation of isocyanic acid over time increases the likelihood of protein carbamylation [51]. Protein solubilization and denaturation with

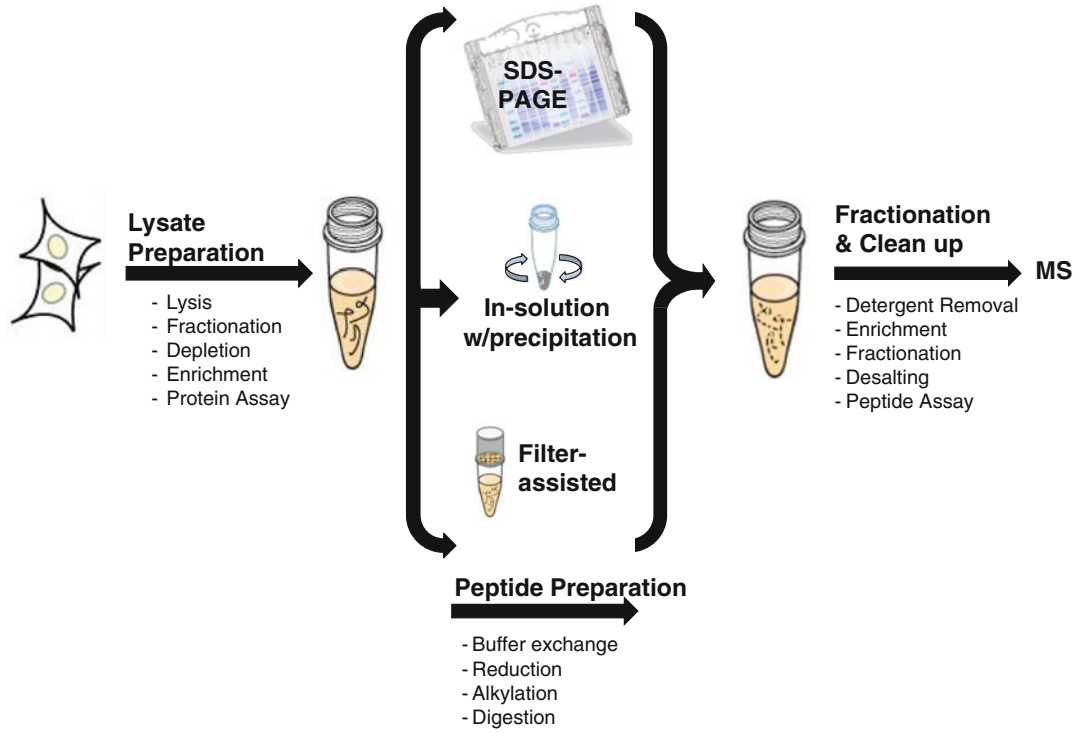

Fig. 3.3 General protein sample preparation workflow. There are many options for the extraction of proteins from tissue and cell lysates, protein fractionation and enrichment, and digestion to peptides for MS analysis

SDS or SDC is more effective than urea, and these detergents permit heating during the reduction of disulfides improving protein denaturation before digestion.

Once disulfides have been reduced and alkylated, contaminating salts, reducing and alkylating reagents, detergents, and small molecule metabolites present in the sample matrix should be removed from the sample before digestion. Depending on the sample source and extraction technique, small molecule contaminants may include excess protein labeling reagents, lipids, nucleotides, and phosphoryl- or amine-containing metabolites (e.g. phosphocholine, aminoglycans, etc.) that could interfere with downstream peptide enrichment or chemical tagging [59]. These contaminants can be removed by buffer exchange using gel filtration resins, dialysis, gel electrophoresis, filtration with a molecular weight cutoff filter, or most commonly by precipitation with an acid or an organic solvent [59–69]. Polydisperse detergents must be removed prior to digestion in order to prevent downstream contamination of LC-MS equipment. Most detergents can be removed by protein precipitation with four volumes of cold (−20 °C) acetone. Precipitation with dilute deoxycholate and trichloroacetic acid, methanol, a 4:1:3 ratio of methanol:chloroform: water, followed by an additional three volumes of methanol, or partitioning with ethyl acetate are alternative methods of detergent removal [65, 67–69]. As an alternative workflow, digestion can be performed in 0.1 % SDS or SDC, and these detergents may be removed from the peptides after digestion using a detergent removal spin column or by acidification to precipitate SDC [52, 70, 71].

Detergents, chaotropes, and organic solvent additives improve trypsin digestion efficiency and dramatically increase peptide and protein identifications in complex protein mixtures [49, 52, 71]. For tryptic digestion, the protein is

dissolved in a buffered solution at pH 8.0 (e.g. 50–100 mM ammonium bicarbonate), and digestion is performed for 4–16 h at 37 °C with agitation. Low concentrations of acetonitrile, urea, SDS, SDC, or MS-compatible detergents may be included to solubilize the precipitated protein pellets and partially denature the protein to improve digestion efficiency. Endoproteinase LysC is an enzyme which cleaves after lysines similar to trypsin. Unlike trypsin, LysC can cleave at lysine residues followed by proline and is active under denaturing conditions (e.g. 8 M urea). LysC digestion is often performed for 1–4 h before tryptic digestion for more complete and reproducible digestion [72]. After digestion, peptides may be desalted off-line using reverse phase solid phase extraction cartridges, tips, or on-line using a trap column before MS analysis, as described in another chapter of this book.

3.8 Peptide Preparation: Filter-Assisted Sample Preparation (FASP)

Molecular weight cutoff (MWCO) filters have been used for decades to concentrate and exchange buffers for protein samples. Protocols for protein sample preparation with MWCO filters prior to MS were introduced in 2005 by Manza et al., and improved upon in 2009 and over subsequent years by the laboratory of Matthias Mann [63, 73, 74]. Filter-assisted sample preparation (FASP) utilizes SDS, heat, and urea to solubilize and denature proteins before transfer to a MWCO spin column which is used for protein collection, concentration, and digestion. An advantage of FASP is that detergents, salts, and small molecules can be easily removed through multiple rounds of washing. Concentrated proteins are then alkylated, washed and digested on the membrane before elution and desalting. FASP is compatible with a wide variety of samples and has been applied to 0.2–200 µg protein samples in a wide variety of applications, including brain tissue samples, formalin fixed paraffin embedded slices, *C. elegans*, phosphoproteomic, and glycoproteomic samples [73, 75–77]. Recently some proposed enhancements to the FASP protocol have been reported including: 1) simultaneous reduction and alkylation to eliminate several centrifugation steps and improve alkylation specificity; 2) prior passivation of the MWCO membrane with Tween-20 for higher peptide recovery, and; 3) the replacement of urea with deoxycholate for improved tryptic digestion [78].

3.9 Peptide Preparation Comparison

As described previously, many proteomic sample preparation methods have been described in the literature (Figs. 3.3 and 3.4), and these methods are modified further by members of the same lab or by other laboratories. This makes it extremely difficult for new MS users to identify the best protocol and generate consistent results. Each of these protocols described here has advantages and disadvantages. GeLC-MS simplifies protein fractionation and maintains peptides from the proteins from a gel band in a single fraction, but it is limited by scale, protein digestion efficiency, and peptide recovery. In-solution digestion with urea can carbamylate lysine residues, requires desalting to remove urea after digestion, and can suffer from poor protein extraction recovery without detergents. FASP is compatible with a wide variety of samples but requires many centrifugation steps, resulting in low sample processing throughput. Finally, digestion in the presence of detergent and subsequent removal of the detergent with a resin, precipitation, or phase transfer extraction may not be scalable or reproducible. Since sample preparation is the most problematic area of MS-based proteome analysis, it is important to have robust, reproducible methods that can be easily adopted by novice and expert MS labs alike.

We have compared the sample preparation results from FASP and three solution-based

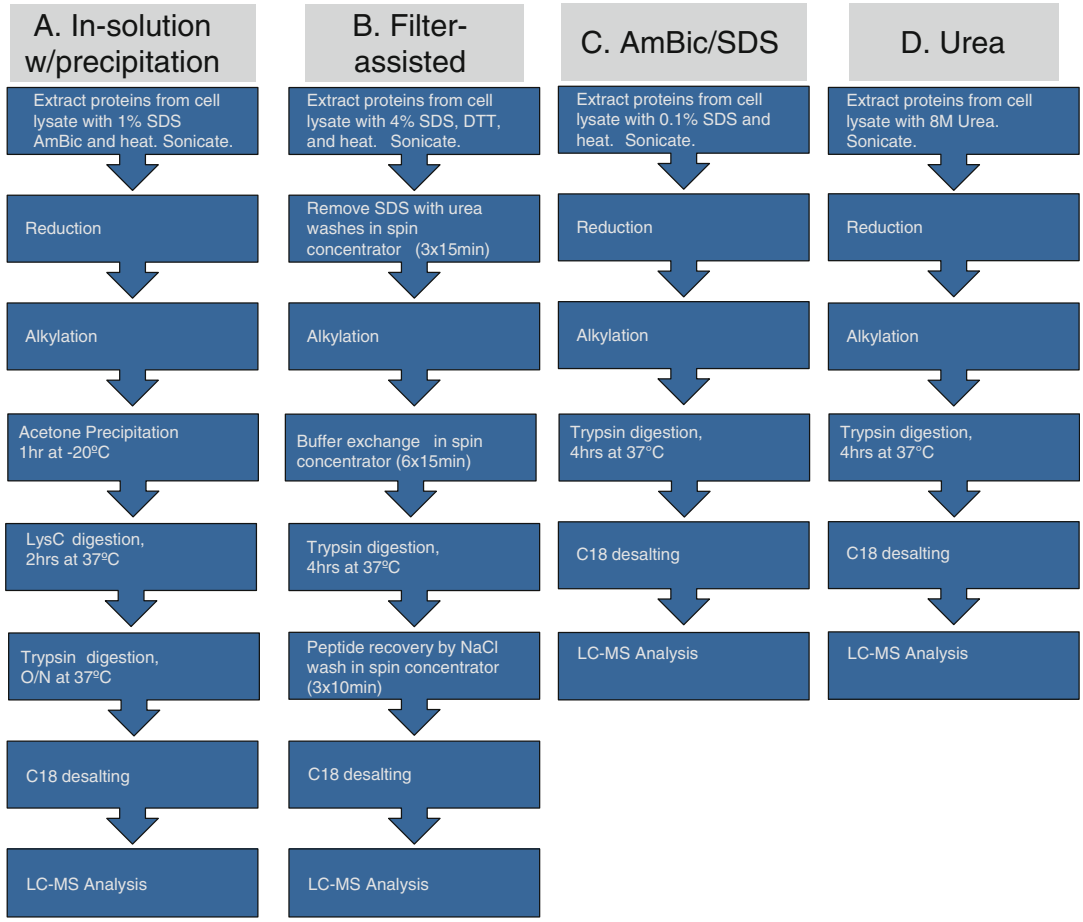

Fig. 3.4 Comparison of standard sample preparation workflows. A summary of the optimized Pierce sample preparation protocol is compared to three other popular standard proteomic sample prep methods that were evaluated

sample preparation methods (Fig. 3.4, [79]). We first used a step-wise approach to optimize a lysis protocol for high protein recovery from mammalian cell lysates. Protein solubilization with 0.1–4 % SDS yielded 5–40 % more protein than solubilization with 8 M urea [79]. Next, the completeness of disulfide reduction, the selectivity of alkylation at cysteine residues, and the digestion efficiency was assessed with single or double digestion (LysC-trypsin) routines. During this analysis, we discovered that improved chromatography resins and columns combined with fast, high resolution instruments often reveal longer, more highly charged peptides with missed cleavages that are not detected on lower resolution or slower mass spectrometers. By optimizing protocols to minimize non-selective alkylation or incompletely digested peptides, we could significantly improve the reproducibility and the number of peptide and protein identifications (Tables 3.1 and 3.2).

Reproducibility of digestion was assessed by the number of identified peptides and proteins identified, by the sequence coverage of a digestion indicator internal standard (Table 3.1), and by the targeted quantitative analysis of peptides from a digestion indicator internal standard. To address this, we spiked a non-mammalian protein in each lysate, processed triplicate samples according to the optimized protocol, and then quantified five peptides by targeted product ion monitoring on a Thermo Scientific Velos ion trap. The coefficients of variation (CV) were

Table 3.1 Reproducibility of LC-MS/MS results from three biological replicates

	Sample 1	Sample 2	Sample 3
Number of Proteins	3382	3228	3376
Number of Unique Peptides	16,333	15,939	17,048
Missed Cleavages (%)	7.8	8.8	8.6
Disulfide Bond Reduction (%)	100	100	100
Cysteine Alkylation (%)	100	100	100
Over Akylation (%)	0.1	0.3	0.9
Digestion Indicator Protein Sequence Coverage (%)	62.50	62.93	65.09

HeLa cell lysate (200 µg) in 200 µL lysis buffer was spiked with 2 µg Digestion Indicator processed by the Pierce Mass Spec Sample Prep Kit for Culture Cells and then analyzed by LC-MS/MS on a Q Exactive mass spectrometer

Table 3.2 Comparison of peptide and protein identification results between sample preparation methods

	Pierce	FASP	AmBic-SDS	Urea
Number of Proteins	3964 ± 22	3894 ± 13	3716 ± 79	3756 ± 91
Number of Unique Peptides	19,902 ± 190	18,738 ± 128	17,401 ± 587	19,398 ± 689
Missed Cleavages (%)	7.3 ± 0.1	13.9 ± 1.2	17.5 ± 1.3	9.8 ± 1.0
Disulfide Bond Reduction (%)	100	100	100	100
Methionine Oxidation (%)	3.0 ± 0.1	11.3 ± 1.5	2.6 ± 0.1	5.3 ± 0.5
Cysteine Alkylation (%)	99.8 ± 0.4	99.8 ± 0.3	100.0 ± 0.0	100.0 ± 0.0
Over Akylation (%)	0.7 ± 0.2	0.1 ± 0.1	0.8 % ± 0.6	2.4 ± 0.4

Hela lysate samples (100 µg) were prepared according to each protocol and 500 ng was analyzed in triplicates by LC-FT MS/IT MS2 CID on an Orbitrap Elite mass spectrometer

4–15 % with a mean CV of 7 % [79]. This quantitative analysis further demonstrated the high reproducibility of sample processing using the optimized protocol.

To assess the scalability of this sample preparation protocol, 10 µg to 5 mg of HeLa cell lysate was processed according to the protocol. Analysis of equivalent volumes of peptide samples by LC-MS/MS resulted in identical chromatograms, demonstrating the scalability of this protocol over a 500x dynamic range of sample amounts (Fig. 3.5). This sample preparation protocol was also used for brain tissue and resulted in reproducible, high quality peptide sample preparations, demonstrating the versatility of this method for different cell and tissue sample types (Fig. 3.6).

We found that the acetone precipitation protocol with optimized reduction, alkylation, and digestion reproducibly yielded high quality peptide samples for LC-MS/MS analysis (Table 3.1). This method yields more protein lysate from cultured cells, is highly reproducible, is scalable, is simpler and faster than FASP, has no risk of carbamylation by urea, and results in higher protein identification rates than other popular "standard" sample preparation methods (Fig. 3.3 and Table 3.2).

3.10 Methods

3.10.1 Protein Extraction

Duplicate or triplicate HeLa S3 cell pellets, each containing 2×10^6 cells, were re-suspended in: (a) 0.2 mL of 0.1 M Tris–HCl, 4 % SDS, 0.1 M DTT, pH 7.6 (FASP method); (b) 0.05 M ammonium bicarbonate, 0.1 % SDS, pH 8.0 (AmBic/SDS method); (c) 0.1 M Tris–HCl, 8 M urea, pH 8.5 (urea method), or (d) Lysis Buffer from the Thermo Scientific Pierce Mass Spec Sample Prep Kit for Cultured Cells. Samples were incubated at 95 °C for 5 min except the urea sample, which was incubated at RT for 30 min. Each cell suspension was sonicated on ice for 20 s. The cell debris was removed by

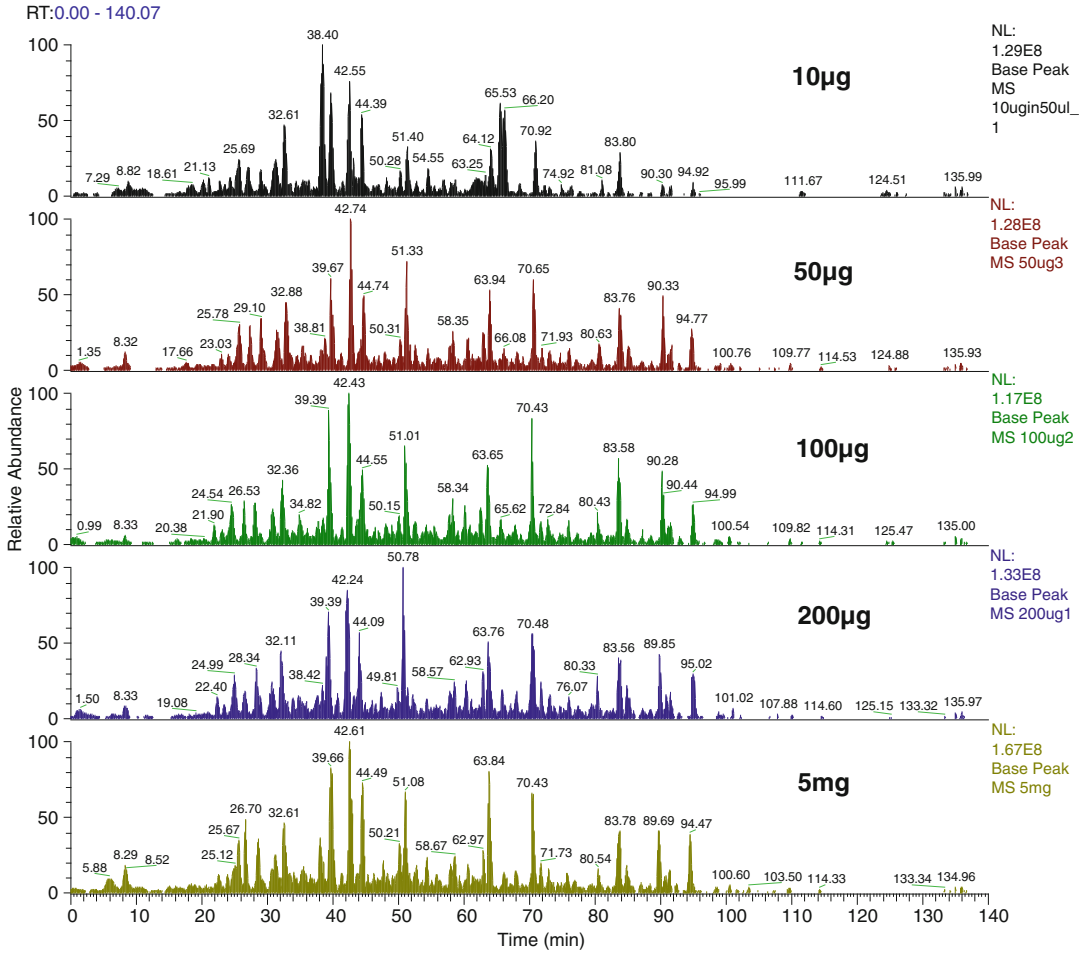

Fig. 3.5 Scalability of new MS sample prep kit protocol. *HeLa* lysate samples (10 µg–5 mg) were prepared according to protocol. Samples (500 ng) subjected to LC-MS/MS analysis on a Thermo Scientific Velos Pro ion trap mass spectrometer

centrifugation at 16,000 × g for 10 min and the supernatant was assayed for protein concentration using Thermo Scientific Pierce BCA Protein Assay or Thermo Scientific Pierce BCA Protein Assay Kit-Reducing Agent Compatible Assay.

3.10.2 Sample Preparation

HeLa cell lysate (100 µg) with digestion indicator (1 %, w/w) was reduced with 10 mM DTT for 45 min at 50 °C and alkylated with 50 mM iodoacetamide for 20 min in dark at RT. Excess iodoacetamide and other contaminants were removed by acetone precipitation at -20 °C for 1 h. The protein was re-suspended in digestion buffer and digested with Lys-C (1:100, enzyme: substrate) for 2 h at 37 °C followed by digestion with trypsin (1:50, enzyme:substrate) overnight at 37 °C. Peptide samples were also prepared according to standard urea, FASP1, and AmBic/SDS workflow.

3.10.3 LC-MS and Data Analysis

A Thermo Scientific EASY-nLC 1000 HPLC system and Thermo Scientific EASYSpray Source with Thermo Scientific EasySpray Column (25 cm × 75 µm i.d., PepMap C18) was

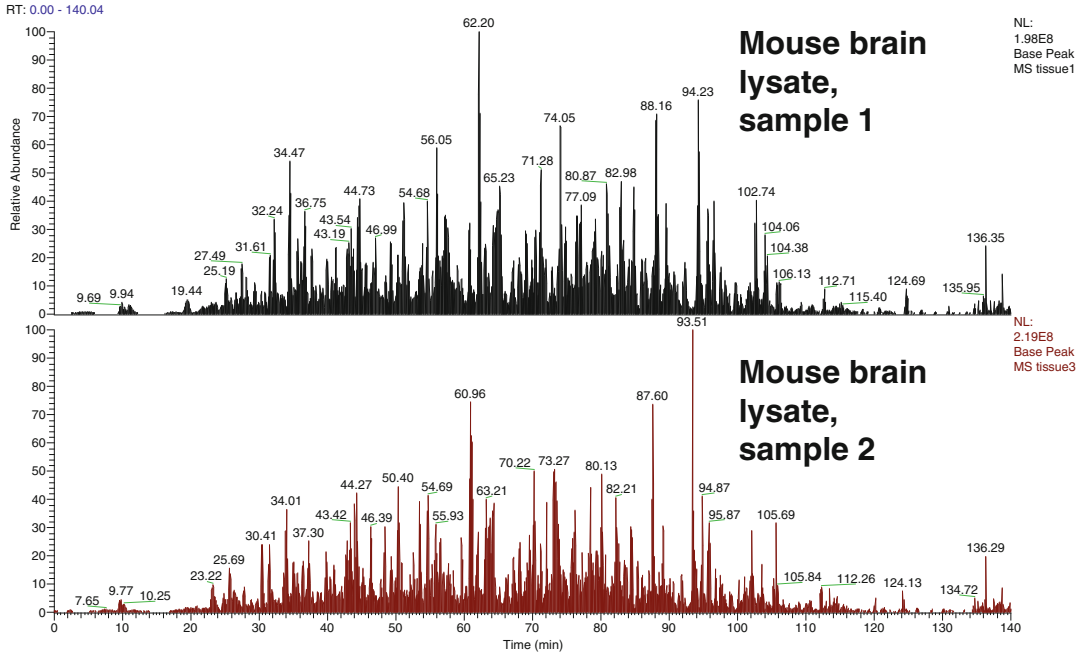

Fig. 3.6 Evaluation of sample preparation workflow with tissue samples. Mouse brain tissue (0.25 g) was homogenized with a tissue tearer and the proteins were extracted using the Thermo Scientific Pierce Mass Spec Sample Prep Kit for Cultured Cells. Tissue lysate (100 µg) was subjected to sample preparation workflow and sample (500 ng) was analyzed by LC-MS/MS on a Thermo Scientific Velos Pro ion trap mass spectrometer

used to separate peptides (500 ng) with a 30 % acetonitrile gradient in 0.1 % formic acid over 100–140 min at a flow rate of 300 nL/min. The samples were analyzed using a Thermo Scientific Velos Pro, a Q Exactive hybrid quadrupole-Orbitrap or an Thermo Scientific Orbitrap Elite mass spectrometers. For data analysis, Thermo Scientific Proteome Discoverer software version 1.4 was used to search MS/MS spectra against the uniprot human database using SEQUEST* search engine with a 1 % false discovery rate. Static modifications included carbamidomethyl (C) and dynamic modifications included oxidation (M). The data set was screened by Preview software (Protein Metrics) for assessment of sample preparation quality. To assess the digestion efficiency, the Digestion Indicator protein sequence was included in the protein database. Five digestion indicator peptides were quantified manually with extracted ion-chromatograms of the raw LC-MS/MS data or automatically with Thermo Scientific Pinpoint 1.2 software.

3.11 Conclusions

A variety of sample preparation methods have been described, along with a brief comparison of several in-solution and filter-assisted sample preparation methods. While each of these methods has advantages and disadvantages, all of these methods are capable of providing contaminant-free peptide samples compatible with mass spectrometric analysis. Unfortunately, none of these sample preparation methods is sufficiently simplified, standardized, or automated to enable rapid adoption and widespread use by novice or non-MS users.

In order to identify thousands of proteins from a complex lysate, it is essential to have robust sample preparation methods for protein extraction, reduction, alkylation, digestion, and clean-up. It is also essential to optimize LC and MS instrument performance, and to regularly (daily or weekly) assess instrument performance with a

standard, well understood positive control samples. A variety of such standards are commercially available, including mixtures of isotopically labeled heavy peptides to assess chromatography, standard digests of common proteins or protein mixtures (e.g. bovine serum albumin and cytochrome C), as well as standard digests of complex proteomes from bacteria, yeast, or human cell lines from several MS reagent vendors. Regular use of standards is critical to ensuring that the instrumentation is working properly before precious samples are analyzed.

Ideally, it would be best to have a simpler, universal sample preparation method, as it would permit standardization of methods and would improve the reproducibility of results across laboratories and over time. For example, decades ago ion exchange-based DNA preparation kits rapidly supplanted the use of ultracentrifugation for plasmid DNA sample preparation. That simplification enabled broader adoption, higher throughput, and standardization of nucleic acid preparation methods. In contrast to DNA extraction from bacteria, the variety of protein sources, the diversity of proteins themselves, and protein biology in general are perhaps too complex to permit similar improvements that simplify, standardize, and automate protein sample preparation. Nevertheless, continued improvements in sample preparation robustness and ease of use are necessary for proteomics methods to be more widely adopted and to successfully advance protein MS beyond academic research or specialized MS labs and into individualized, bench top point of use or large clinical applications.

Supplementary Protocols

1. In-gel Digestion

Materials

- 25 mM ammonium bicarbonate: Dissolve 80 mg ammonium bicarbonate in 40 mL ultrapure water
- Destain solution: 25 mM ammonium bicarbonate/50 % acetonitrile (ACN). Mix 80 mg of ammonium bicarbonate with 20 mL of acetonitrile (ACN) and 20 mL of ultrapure water.
  Note: if destaining glutaraldehyde-free silver stained gels, prepare separate 100 mM sodium thiosulfate and 30 mM potassium ferricyanide solutions, then make destaining solution by mixing them in a 1:1 (v:v) ratio. Protect ferricyanide solution from light.
- DTT stock solution: 10 mM DTT in 25 mM ammonium bicarbonate
- Iodoacetamide (IAM) stock solution: 20 mM in 25 mM ammonium bicarbonate (always prepare fresh, protect from light)
- 10 ng/μl Trypsin, sequencing-grade (use 25 mM ice cold ammonium bicarbonate to dilute stock trypsin solution, immediately before adding to gel pieces)

Equipment

- Gloves! (to minimize keratin contamination)
- Clean glass plate (large enough to place entire gel on and room for a working area, 8" × 8")
- Gel-cutting devices: clean steel razor blades or surgical scalpel
- Low protein binding micro-centrifuge tubes (0.65 mL or 1.5 mL)
- Gel-loading pipette tips
- Autosampler vials with perforated caps
- SpeedVac Concentrator

Sample Processing

1. Place the gel on a clean glass plate. Cover the gel with just enough ultrapure water to prevent dehydration during the slicing process.
2. Cut the gel lane using (new, if possible) scalpel or razor blade.
3. Cut each of the excised bands into 1–2 mm cubes and transfer these cubes to a 0.65 mL low protein binding microcentrifuge tube.
4. Add ~100 μL (or enough to cover gel slices) of 25 mM ammonium bicarbonate/50 % ACN and vortex for 10 min.

5. Using gel loading pipet tip, extract the supernatant and discard. The procedure should be repeated until the stain is completely removed. Two additional washes should be sufficient for moderately intense bands.
6. Add 100 μL of 5 mM DTT and incubate for 30 min at 50 °C. Spin. Discard all the liquid afterwards.
7. Allow samples to cool to room temperature.
8. Add 100 μL of 20 mM iodoacetamide and incubate the gel pieces in the dark for 45 min at room temperature. Spin. Discard the liquid afterward.
9. Wash the gel pieces with 100 μL of 25 mM ammonium bicarbonate, vortex 10 min, spin. Discard the liquid afterwards.
10. Wash the gel pieces with ~100 μL (or enough to cover) of 25 mM ammonium bicarbonate in 50 % ACN, vortex 10 min, spin. Discard the liquid.
11. Dehydrate the gel pieces in 100 % ACN for 10 min, spin and discard the liquid afterwards.
12. Dry the sample in a speed-vac for 10 min. The gel pieces are now ready for tryptic digestion.
13. Just before use, dilute or reconstitute trypsin with 50 mM ice cold ammonium bicarbonate to give final concentration of the 10 ng/μL.
14. Add trypsin solution to just cover the gel pieces.
15. Verify that the gel pieces are covered with trypsin solution.
16. Add 25 mM ammonium bicarbonate as needed to cover the gel pieces.
17. Spin briefly and incubate at 37 °C for 4 h – overnight.
18. Stop digestion by adding 20 μL of 5 % formic acid.
19. Vortex 15–20 min, spin, and transfer the digest solution (aqueous extraction) into a clean autosampler vial appropriate for LC/MS-MS.
20. To the gel pieces, add 30 μL (enough to cover) of 50 % ACN/1 % formic acid, vortex 15–20 min., spin, and transfer solution to the tube used above. Repeat this step once.
21. Concentrate peptide extracts using a speed-vac concentrator to a volume that is slightly larger than will be used for injection during LC-MS/MS analysis.
22. Store the vial with the extracted peptides at −20 °C if the samples will not be run the same day.

2. In-Solution Sample Preparation With Acetone Precipitation

Materials

- 100ABCS: 100 mM NH4HCO3 with 0.1 % sodium dodecyl sulfate, pH 8.0, 5 mL
- 50ABC: 50 mM NH4HCO3, pH 8.0, 5 mL
- 500 mM DTT in 50ABC, 0.5 mL
- 500 mM Iodoacetamide (IAM) in 50ABC, 0.5 mL (protect solution from light)
- 0.1 % acetic acid in water, 250 μL
- Lys-C Protease, MS Grade, 20 μg
- MS-Grade Trypsin Protease, MS Grade, 20 μg
- Pre-chilled 90 % acetone: Prepare 90 % acetone in ultrapure water (e.g., mix 45 mL of 100 % acetone with 5 mL of ultrapure water) and store at −20 °C.
- Pre-chilled 100 % acetone: Store 100 % acetone at −20 °C.
- Trifluoroacetic acid (TFA)
- Phosphate-buffered saline (PBS)

Equipment

- Low protein binding microcentrifuge tubes
- Microtip probe sonicator or nuclease (e.g., Thermo Scientific™ Pierce™ Universal Nuclease for Cell Lysis, Product No. 88700)
- Heating block
- SpeedVac Concentrator

Procedure

Cell Lysis

1. Culture cells to harvest at least 100 μg of protein. For best results, culture a minimum of 1×10^6 cells.

Note: Rinse cell pellets 2–3 times with 1X PBS to remove cell culture media. Pellet cells using low-speed centrifugation (i.e., < 1000 × g) to prevent premature cell lysis.

2. Lyse the cells by adding five cell-pellet volumes of 100ABCS (i.e. 100 µL of 100ABCS for a 20 µL cell pellet). Pipette sample up and down to break up the cell clumps and gently vortex sample to mix.
3. Incubate the lysate at 95 °C for 5 min.
4. Cool the lysate on ice for 5 min.
5. Sonicate lysate on ice using a microtip probe sonicator to reduce the sample viscosity by shearing DNA.
6. Centrifuge lysate at 14,000 × g for 10 min at 4 °C.
7. Carefully separate the supernatant and transfer into a new tube.
8. Determine the protein concentration of the supernatant using established methods such as the BCA Protein Assay Kit

Reduction, Alkylation and Acetone Precipitation

Note: This procedure is optimized for 100 µg of cell lysate protein at 1 mg/mL concentration; however, the procedure may be used for 10–200 µg of cell lysate protein with an appropriate amount of reagents (DTT, IAM, Lys-C and trypsin). When using 10 µg of cell lysate, a protein concentration of 0.2–1 mg/mL may be used.

1. Add 100 µg of lysate protein to a polypropylene microcentrifuge tube and adjust the sample volume to 100 µL using 100ABCS to a final concentration of 1 mg/mL.
2. Add 2.1 µL of DTT solution to the sample (final DTT concentration is ~10 mM). Mix and incubate at 50 °C for 45 min. Discard any unused DTT solution.
3. Cool the sample to room temperature for 10 min.
4. Add 11.5 µL of IAM solution to the sample (final IAM concentration is ~50 mM). Mix and incubate at room temperature for 20 min protected from light. Discard any unused IAM solution.
5. After alkylation with IAM, immediately add 460 µL (4 volumes) of pre-chilled (−20 °C) 100 % acetone to sample. Vortex tube and incubate at −20 °C for 1 h to overnight to precipitate proteins.
6. Centrifuge at 14,000 × g for 10 min at 4 °C. Carefully remove acetone without dislodging the protein pellet.
7. Add 50 µL of pre-chilled (−20 °C) 90 % acetone, vortex to mix and centrifuge at 14,000 × g for 5 min at 4 °C.
8. Carefully remove acetone without dislodging the protein pellet. Allow the pellet to dry for 2–3 min and immediately proceed to Protein Digestion.

   Note: Do not dry the acetone-precipitated protein pellet for more than 2–3 min; excess drying will make the pellet difficult to re-suspend in the Digestion Buffer.

Enzymatic Protein Digestion

9. Add 100 µL of 50ABC to the acetone-precipitated protein pellet and resuspend by gently pipetting up and down to break the pellet.

   Note: An acetone-precipitated protein pellet may not completely dissolve; however, after proteolysis at 37 °C, all the protein will be solubilized.
10. Immediately before use, add 40 µL of ultrapure water to the bottom of the vial containing lyophilized Lys-C and incubate at room temperature for 5 min. Gently pipette up and down to dissolve. Store any remaining 0.5 µg/µL Lys-C solution in single-use volumes at −80 °C.
11. Add 2 µL of Lys-C (1 µg, enzyme-to-substrate ratio = 1:100) to the sample. Mix and incubate at 37 °C for 2 h.
12. Immediately before use, add 40 µL of 0.1 % acetic acid to the bottom of the vial containing trypsin and incubate at room temperature for 5 min. Gently pipette up and down to dissolve. Store

any remaining 0.5 μg/μL trypsin solution in single-use volumes at −80 °C for long-term storage.

13. Add 4 μL of trypsin (2 μg, enzyme-to-substrate ratio = 1:50) to the sample. Mix and incubate overnight at 37 °C.
14. Freeze samples at −80 °C to stop digestion. (Optional: stop digestion by acidifying with TFA)
15. Speed vac sample to 1–5 μL.
16. Resuspend the sample in an appropriate buffer (e.g., 0.1 % TFA) for LC-MS analysis.
    Note: Proteolytic digests prepared using this protocol are directly compatible with LC-MS analysis. Clean-up of samples with C18 spin tips or columns is optional.

3. Filter-assisted Sample Preparation (FASP)

Materials

- UABC: 8 M urea in 100 mM NH_4HCO_3 (ABC) pH 8.0. Prepare fresh, 1 mL per sample.
- IAM solution: 55 mM iodoacetamide in UABC. Prepare 100 μL per sample.
- DTT solution: 50 mM DTT in UABC. Prepare 100 μL per sample
- Trypsin: MS grade Modified Trypsin, 0.5 μg/μL in 50 mM NH_4HCO_3 in water
- 50ABC: 50 mM NH_4HCO_3 in water. Prepare 0.5 mL per sample
- 25ABC: 25 mM NH_4HCO_3 in water. Prepare 0.25 mL per sample
  Note: UABC and IAM solutions must be freshly prepared and used within a day. IAM is light sensitive, so protect from light

Equipment

- Low protein binding tubes
- 10 or 30 kDa cut off filter (Vivacon 500, cat # VN01H02)
- Bench-top centrifuge
- Temperature-controlled incubator or heat block at 50 °C
- Thermo-mixer at 37 °C
- SpeedVac Concentrator

Procedure

1. Combine up to 30 μL of a protein extract (0.2–400 μg) with 200 μL of UABC in the filter unit and centrifuge at 14,000 × g for 15 min.
2. Add 200 μL of UABC to the filter unit and centrifuge at 14,000 × g for 15 min.
3. Discard the flow-through from the collection tube.
4. Add 100 μL DTT solution and mix at 600 rpm in a thermo-mixer for 1 min and incubate at 50 °C without mixing for 45 min.
5. Centrifuge the filter units at 14,000 × g for 10 min.
6. Add 100 μL IAM solution, cover with foil, mix by gentle vortexing for 1 min, and incubate in dark at room temperature without mixing for 30 min.
7. Centrifuge the filter units at 14,000 × g for 10 min.
8. Add 100 μL of UABC to the filter unit and centrifuge at 14,000 × g for 15 min. Repeat this step one more time.
9. Add 100 μL of 50ABC to the filter unit and centrifuge at 14,000 × g for 10 min. Repeat this step one more time.
10. Transfer the filter units to new collection tubes.
11. Add 100 μL of 50ABC with trypsin (enzyme to protein ratio 1:50) and mix at 600 rpm in thermo-mixer at 37 °C for 4–18 h.
12. Centrifuge the filter units at 14,000 × g for 10 min.
13. Add 50 μL of 25ABC and centrifuge the filter units at 14,000 × g for 10 min.
14. Add 50 μL of 10ABC and centrifuge the filter units at 14,000 × g for 10 min.
15. Concentrate down to ~5 μL and add 0.1 % FA to a final volume of ~20–25 μL.

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