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Facile Preparation of Peptides for Mass Spectrometry Analysis in Bottom-Up Proteomics Workflows

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Abstract

Mass spectrometry (MS) is routinely used to identify, characterize, and quantify biological molecules. For the analysis of proteins, MS-based workflows can be broadly categorized as top-down or bottom-up, depending on whether the proteins are analyzed as intact molecules or are digested into peptides prior to MS, respectively. This article outlines the steps required to prepare peptide samples for MS within the context of a bottom-up proteomics workflow. The bottom-up proteomics methods described are versatile and can be used for discovery and targeted analyses in qualitative and quantitative workflows. Samples resulting from these workflows contain peptides of suitable size for analysis by MS instrumentation most commonly available to modern research laboratories, including MS coupled to either liquid chromatography (LC) or matrix-assisted laser desorption/ionization (MALDI) interfaces. Numerous bottom-up sample preparation strategies are available and the number of protocols and variations in experimental detail can be overwhelming for users without prior experience in proteomics. This article thus incorporates recent developments in methodologies and consumables that make sample preparation more facile than ever before, and the protocols are well-suited to users without prior experience in proteomics. Included are protocols for universally applicable suspension trapping processing, as well as an alternate in-solution processing to accommodate a range of sample types. Protocols for removing detergents, polymers, and salts from peptide samples, performing protein and peptide quantification, and fractionating peptides according to hydrophobicity prior to MS analysis are also described.

Keywords

Bottom-up proteomics; S-Trap™; detergent removal; mass spectrometry

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CONFLICTS OF INTEREST:

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DATA AVAILABILITY STATEMENT

The data that support the findings will be available in MassIVE (https://massive.ucsd.edu; MSV000086814) following an embargo from the date of publication to allow for commercialization of research findings.

INTRODUCTION:

Mass spectrometry (MS) is routinely used to identify, characterize, and quantify biological molecules. In the past 20 years, numerous MS-based proteomics methods have been developed to determine protein identity, quantity, interaction partners, and the presence and localization of post-translational modifications. MS-based proteomics workflows can be broadly categorized as top-down or bottom-up (Kelleher et al., 1999). Top-down approaches involve the MS analysis of intact proteins to obtain amino acid sequence information in a way that preserves the stoichiometry among post-translational modifications, proteolytic cleavage products, and products of splicing events. Bottom-up approaches, on the other hand, involve the enzymatic or chemical digestion of proteins into peptides prior to MS analysis, and can be used to identify and quantify peptides, which are then used to infer protein identity and quantity. The presence and sites of post-translational modifications can also be determined. While top-down proteomics methods offer the advantage of preserving the relationship between the protein sequence and modifications, bottom-up strategies remain among the most popular strategies for the analysis of biological samples. The popularity of bottom-up methods is tied to the versatility of the approaches, availability of instrumentation, and bioinformatic tools for data analysis (Mayne et al., 2016). Briefly, bottom-up methods can be used for discovery and targeted analyses in qualitative and quantitative workflows. In addition, samples resulting from bottom-up workflows contain peptides of suitable size for analysis by MS instrumentation most commonly available to modern research laboratories. Finally, many open-source and commercially developed software tools are available to support data analysis for a broad range of experimental designs (Bruce et al., 2013; Chen et al., 2020).

Bottom-up strategies can be used to identify and quantify thousands of proteins from a single sample, all without prior knowledge of the sample composition or reliance on antibodies. Bottom-up workflows typically involve a series of steps to digest the protein into peptides either by enzymatic or chemical digestion, followed by removal of contaminants before analysis by MS (see Fig. 1A for general overview of the workflow). Once high-quality tandem mass spectrometry (MS/MS) data are recorded for the peptides, modern bioinformatics approaches can be used to determine peptide and inferred protein identity, sites of post-translational modifications, and, with an appropriate experimental design, relative abundance of peptides among samples (Tsiamis et al., 2019).

Many different types of samples, including purified proteins, cells, tissues, and biological fluids can be used as starting material for bottom-up workflows. Recent developments in sample preparation methodologies and consumables make it easier than ever to prepare high-quality samples from various types of starting material. Since publishing our previous article describing the preparation of protein from polyacrylamide gels (Gundry et al., 2009), the field has increasingly favored gel-free approaches because of advantages in time, ease, and minimization of sample loss. Also, whereas previous methods have included protein precipitation as a method to remove detergents and other contaminants from protein samples, the use of suspension trapping allows proteins to be solubilized in harsh detergent and directly digested without precipitation. Finally, while traditional peptide cleanup methods using reversed-phase chromatography could be used for desalting but not detergent

or polymer removal, new approaches that use carboxylate coated magnetic particles achieve the needed removal of detergents, polymers, and salts.

Numerous bottom-up sample preparation strategies are available and the number of protocols and variations in experimental detail can be overwhelming for users without prior experience in proteomics. This article outlines the basic steps required to prepare samples for MS analysis within the context of a bottom-up proteomics workflow. The protocols take advantage of new developments in methodologies and consumables to make sample processing easier. Also, guidance for selecting the appropriate method for various types of biological samples is provided. The choice of bottom-up workflow will depend on sample complexity and goals of the experiment. Fig. 1A outlines the major steps in a bottom-up workflow and Fig. 1B and 1C illustrate how the Basic and Support Protocols described here can be mixed and matched together into a seamless workflow tailored to the experimental goals. Sample complexity (*i.e.* the number of proteins and dynamic range of protein concentration) and relative ease in which the sample can be solubilized will determine whether a workflow involving rigorous homogenization followed by suspension trapping using an S-trap™ column (Basic Protocol 1) or an in-solution processing workflow (Alternate Protocol 1) should be used. A fast and easy method for detergent and salt removal from peptide mixtures (Alternate Protocol 2) is effective for cleaning samples generated by Alternate Protocol 1 and can also be applied to samples generated by virtually any other bottom-up workflow described elsewhere. Basic Protocol 1, on the other hand, includes sample cleanup as part of its protocol by nature of the S-trap™ column. Although protein and peptide quantification prior to MS analysis are not universally required for all bottom-up experiments, we strongly urge their inclusion when sufficient sample amount is available. Accurate protein quantification (Support Protocol 1) is useful for calculating how much enzyme or chemical is required for digestion. It is also important for selecting the column size with suitable capacity in the S-trap™ processing step (Basic Protocol 1) as well as ensuring sufficient volume is used to solubilize proteins in the in-solution processing workflow (Alternate Protocol 1). Peptide quantification (Support Protocol 2) is useful for determining whether the peptide sample should be diluted prior to MS analysis, to avoid overloading the liquid chromatography (LC) column used for separation in LC-MS analyses. Peptide quantification is essential in quantitative workflows where equivalent amounts of total peptide are compared to reveal differences in relative abundance of individual peptides. Finally, the peptide mixtures generated by Basic Protocol 1 or Alternate Protocol 1 may be fractionated by separating peptides according to their hydrophobicity (Support Protocol 3) to enhance coverage of the proteome for moderate to high complexity samples, as lower abundance proteins may be more difficult to detect in these sample types.

STRATEGIC PLANNING

Protein samples for bottom-up MS analysis can be prepared from a variety of sources, and the complexity of the sample should be considered when choosing between Basic Protocol 1 or Alternate Protocol 1. Low complexity samples are defined here as purified proteins and mixtures containing hundreds of proteins. These types of samples commonly result from affinity purification or reversed-phase high performance liquid chromatography (RP-HPLC) used to concentrate or purify intact proteins. Moderate complexity samples are those with

several thousand proteins and include whole cell lysates or subcellular fractions, while high complexity samples are those with several thousand proteins from multiple cell types, such as tissue. Biological fluids such as plasma are also considered highly complex in this context because they can contain many non-protein substances (*e.g.* lipids, small molecules), which can pose challenges to sample preparation, and can exhibit protein concentrations spanning 12 orders of magnitude (Anderson and Anderson, 2002). Alternate Protocol 1 is suitable for low to moderate complexity samples where the proteins of interest are readily soluble in the digestion buffer used in this protocol (*i.e.* aqueous/organic solution containing MS-compatible surfactant). Therefore, it is advisable to first perform a solubilization test to ensure the sample can be solubilized in this buffer. For example, we find that various cell types (*e.g.* B lymphocytes, HEK293T, cardiac fibroblast, and HeLa cells) can be processed with Alternate Protocol 1. However, cells that contain a high content of insoluble cytoskeletal proteins such as muscle cells, perform better in Basic Protocol 1. Importantly, for cultured cells, no specific culture conditions are required. Rather, Basic Protocol 1 is generalizable to any culture conditions necessary to address the biological question of interest.

There is no absolute lower or upper limit to the starting amount that can be processed by Alternate Protocol 1. Generally, a larger amount of protein present in the starting sample will yield more peptide identifications by MS. However, for Basic Protocol 1, each S-trap™ column has a defined optimal binding capacity. Therefore, it is advisable to determine how much protein is in a sample when planning an experiment, to maximize peptide and inferred protein identifications. For sample-limited cases, it is recommended to perform protein quantification (Support Protocol 1) on a representative sample (*e.g.* from a control that is not sample-limited) to provide an estimate of the amount of protein that will be available from the experimental sample. If using cells, it is advised that the number of cells to be used for the experiment are counted ahead of time (*e.g.* via manual or automated counting of trypan blue excluded cells) to provide a general guide as to the expected amount of total protein present. It is recommended to begin with at least 10 μg of protein to recover enough peptides after processing for successful identification by most modern MS/MS instruments. If protein and peptide quantification (Support Protocols 1 and 2) will be performed, it is recommended to start Alternate Protocol 1 with 50 μg protein, so that there is enough material to be used for these quantification assays. The upper limit is defined by solubility and maximum volume of the tube. If 1.5 mL microfuge tubes are used, this practically limits the digestion volume to 1.2 mL, which is typically sufficient for at least 2500 μg of protein.

Basic Protocols 1 and Alternate Protocol 2 are described for trypsin/lysC digestion at pH 8.0. If another enzyme is used, the pH should be adjusted according to the corresponding manufacturer's guidelines.

Basic Protocol 1 is described for a spin column format but can be adopted to higher throughput analyses using the S-Trap™ spin column plate format following the manufacturer's recommendations for sample loading amounts and solution volumes.

Prior to starting Alternate Protocol 2, it is recommended to perform Support Protocol 2 to determine the concentration of peptides in the sample so that the mass of total peptide for

cleanup can be inferred. The mass of total peptide will be used to determine the volume of particle solution needed to clean the peptide sample. When performing Support Protocol 2, recovery is maximized using 0.2–0.4 μL of 50 μg/μL particle suspension per μg of peptide (representing a 10:1 to 20:1 particle:peptide ratio). Over 80% recovery has been observed but it is recommended to conservatively estimate that 50% of peptides will be recovered and, therefore, the amount of peptide desired for MS analysis will determine how much peptide sample to cleanup.

BASIC PROTOCOL 1: PREPARATION OF HIGH COMPLEXITY PEPTIDE SAMPLES FOR MASS SPECTROMETRY ANALYSIS USING S-TRAP™ PROCESSING.

This protocol outlines the steps required to prepare high complexity samples for MS analysis by suspension trapping using an S-Trap™ column (Fig. 1B and 2). This protocol is suitable for processing a wide variety of sample types, and details are provided for processing cells and tissues. By omitting the homogenization and sonication step used for cells and tissues, the workflow can be adopted for plasma, serum, urine, and cerebral spinal fluid, as described elsewhere (Ding et al., 2020; Mindikoglu et al., 2020). The protocol describes how to homogenize the sample in 5% sodium dodecyl sulfate (SDS), then immobilize, reduce, alkylate, clean up, and digest the proteins on the S-Trap™ column. Peptides are then eluted from the column and can be analyzed directly by MS. Alternatively, for samples where enhanced sequence coverage of lower abundance peptides is required, Support Protocol 3 can be optionally used to fractionate the mixture of peptides based on their hydrophobicity (Fig. 1B). Finally, Support Protocol 2 can be used directly after Basic Protocol 1 or Support Protocol 3 to inform how much of the sample should be used for MS analysis, or to normalize total peptide in the case of quantitative analyses.

NOTE: Use only HPLC and/or LC-MS grade solvents throughout the protocol. All steps are performed at room temperature unless otherwise noted.

Materials:

S-Trap™ micro columns (≤ 100 μg, 10 pack, Protifi, PN C02-micro-10)

Precellys lysing kit (Precellys, PN P000926-LYSK0-A), or other homogenizing bead tubes

Reagents and equipment for peptide and protein quantification (see Support Protocols 1 and 2)

Phosphoric acid (85%, ACS grade, Thermo Fisher Scientific, PN A260)

Trypsin/LysC (Promega, PN V5071)

Purified water (18 MΩ)

12% phosphoric acid (for pH of solvents as well as S-Trap™ protocol) (see Reagents and Solutions).

Lysis Buffer: if using tissue, SDS Tissue lysis buffer (see Reagents and Solutions); if using cells, SDS Cell lysis buffer (see Reagents and Solutions)

Benzonase®Nuclease (Sigma Aldrich, PN E1014–25KU) (optional)

400 mM acrylamide (Sigma, PN A3553)

100 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 M TCEP, Sigma, PN C4706)

S-Trap™ binding/wash buffer (see Reagents and Solutions)

S-Trap™ digestion buffer (50 mM TEAB in LC-MS grade water)

10% formic acid (FA) (FA, LC-MS grade, Fisher Scientific, PN A117) in LC-MS water (see Reagents and Solutions)

0.2% FA in LC-MS grade water (see Reagents and Solutions)

0.2% FA in 50% LC-MS grade acetonitrile (Fisher Scientific, PN A955) (see Reagents and Solutions)

0.1% FA in 2% LC-MS grade acetonitrile (see Reagents and Solutions)

50 mL conical centrifuge tubes (Fisher Scientific, PN 06-443-19) (for solution storage)

20 mL disposable glass vials (for solution storage)

Low retention 1.5 mL microfuge tubes (Thermo Fisher Scientific, PN 02-681-320)

10 μL glass syringe or other glass pipette device

Variety of positive displacement pipettes (*e.g.* P10 – P1000)

Low retention pipette tips (*e.g.* 0.5 μL – 1 mL)

15 mL conical tubes

Dissection tweezers

Razor blade

Homogenizer (*e.g.* Bertin Instruments, Precellys 24)

Block sonicator (*e.g.* Hielscher, UP200St)

Refrigerated centrifuge

Ice bucket with lid (can alternatively use aluminum foil to cover ice bucket)

pH meter

Microcentrifuge with adjustable rotor speed up to 7,000 × g

Mixing incubator (*e.g.* Thermomixer)

Vortexer

Vacuum centrifuge (*e.g.* SpeedVac)

Protocol Steps

Homogenize and Sonicate Tissue or Cells

1a. If using tissue: Dice flash-frozen or fresh tissue with razor in a disposable weigh dish. Add approximately 50–100 mg of tissue into a tared Precellys 2 mL bead beater tube. Record weights.

Dice frozen tissue while still frozen. Keep samples on ice throughout protocol unless stated otherwise to reduce enzymatic digestion.

Process as follows:

i. Add 9x the tissue weight in mL of SDS tissue lysis buffer to the sample. For example, for 50 mg tissue, add 450 mL of SDS buffer. Cap the tube tightly.

ii. Use the PreCellys bead beater 24 or other homogenizer to homogenize the samples at 6500 rpm, 2 times at 15 seconds each, with 10 seconds pause. Immediately place tubes on ice.

1b. If using cells: The amount of lysis buffer to use is based on the number of cells. Into the tube that contains the pelleted cells, add the appropriate amount of SDS cell lysis buffer and Benzonase to degrade DNA using the information below as a guide.

Number of cells	1–2 million	5 million	10 million	15–20 million
Lysis buffer	250 µL	300 µL	500 µL	600–800 µL
Benzonase	0.5 µL	0.5 µL	1 µL	1.5 µL

2. Place sample tube from Step 1a or 1b in the block sonicator's tube adaptor that is suspended above an ice bucket. Make sure that both the bottom and top of the tube are embedded in ice. Run for 10 seconds on and then 10 seconds off, 3 times at 30% power. Wait 1 minute and repeat the cycle two more times. Keep on ice afterwards.

Sonication is necessary in order to shear DNA within the sample, which would otherwise clog the S-Trap™. Optimize the power levels according to your sonicator. While a probe sonicator may be used, this increases the chance for cross-contamination, as the probe is reused and requires careful cleaning between uses. The block sonicator used here enables samples in a closed tube to be processed without cross-contamination.

3. Clarify the sample by centrifugation for 10 minutes at 13,000 × g at 4 °C. If there is visible particulate of insoluble material, use a P100 pipette to aspirate at least 100 µL of cell lysis off the top without disturbing particulate at the bottom of the tube, and transfer to a clean centrifuge tube.

4. Perform protein quantification. See Support Protocol 1.

Determining the amount of protein in the sample will inform the amount of enzyme to use in later steps. This information can also be used to normalize among samples intended for quantitative analyses. As stated in the Strategic Planning section, for sample-limited cases, it is advisable to have an estimate of the total amount of protein based on a similar sample type, when possible. If, based on a representative sample, the amount of protein in the experimental sample is less than 10 μg, it will not be possible to perform protein quantification without using a majority of the sample for this step. However, if starting with 50–100 mg tissue or 5 million cells as stated above, there will be sufficient material to perform protein quantification.

Reduce disulfide bonds and alkylate free cysteine residues

6. If the protein concentration exceeds 2 μg/μl, dilute samples to 2 μg/μl with lysis buffer and transfer 25 μL (i.e. 50 μg of protein) to a new tube.

It is possible to use more than 25 μL, but this would require additional loading steps onto the S-trap™.

7. Reduce disulfides by adding 2.5 μL of 100 mM TCEP (to a final concentration of 10 mM TCEP). Cap and vortex to mix.

8. Place tube(s) on an incubating mixer at 1200 rpm at 37°C for 30 minutes.

9. Alkylate cysteines by adding 2.75 μL 400 mM acrylamide (to a final concentration of 40 mM). Cap and vortex to mix.

10. Place tube(s) on the incubating mixer at 1200 rpm at 37°C for 30 minutes.

Immobilize and Digest Proteins

11. Add 3 μL of 12% phosphoric acid. Cap and vortex to mix.

This step is essential as it brings the protein to the appropriate pH for trapping on the S-Trap™. Sample will be lost if this step is not performed. If starting with more volume than presented in these steps, add at least 10% volume.

12. Add 165 μL of S-Trap™ binding/wash buffer to the 33 μL of acidified sample from Step 10 giving a total volume of ~198 μL. Cap and vortex to mix. Protein particulate may form initially but solution should appear translucent after vortexing. Do not centrifuge.

Centrifuging the sample at this step increases the risk of precipitating proteins and increases sample loss.

13. Place the S-Trap™ column in a 2.0 mL microfuge tube and add the acidified sample mixture into the S-Trap™ column.

Column equilibration is not needed. Do not load more than 200 µL to the spin column. Do not allow the waste volume at the bottom of the microfuge tube to rise above the level of the bottom surface of the S-Trap™ column.

14. Centrifuge the S-Trap™ column for 2 minutes at 4,000 × g or until all of the sample has passed through the S-Trap™ column. Discard flow through.

15. Wash the S-Trap™ column by adding 150 µL of S-Trap™ binding/wash buffer to each trap. Repeat centrifugation. Discard flow through.

The use of a centrifuge with swinging buckets is ideal. However, if not available, a fixed angle centrifuge can be used. If using fixed angle, rotate the S-Trap™ columns a half turn between the centrifugation steps to avoid asymmetrical packing and washing. Remove wash flow-through as necessary (i.e. after two centrifuge cycles for 2 mL waste tube, but every cycle for smaller) or transfer to additional tubes. Vacuum manifolds that apply pressure may also be used.

16. Move the S-Trap™ column to a clean low retention 1.5 mL microfuge tube for the digestion.

17. Add 100 µl of digestion buffer to one vial of 20 µg trypsin/lysC. Add 20 µL of the resulting dilution to the top of the S-trap™ column.

It is important to verify there is no air bubble between the digestion solution and the trap bed by placing the tip of the pipette directly on the S-Trap™ sorbent bed when dispensing. The S-Trap™ will immediately begin to absorb the solution but may not flow completely. Any air bubbles may be removed by gently tapping the side of the spin column. This protocol has been optimized for a complex sample with a total protein amount of 50 µg and uses an enzyme:protein ratio of approximately 1:12.

18. Cap the S-Trap™ column to limit evaporative loss.

19. Proceed with trypsin/lysC digestion by incubating the sample for 2 hours at 37°C without agitation.

20. Uncap the samples and add 40 µL of 50 mM TEAB digestion buffer.

21. Centrifuge the S-Trap™ column in the sample collection tube for 2 minutes at 4,000 × g.

The eluted peptides will collect in the sample tube. Do not discard this tube as you will do three elutions and all of them will be collected in the same tube.

22. Elute again into the same tube with 40 µL of 0.2% formic acid in water. Centrifuge again at 4,000 × g for 2 minutes.

23. Elute hydrophobic peptides into the same tube with 35 µL of 0.2% formic acid in 50% acetonitrile. Centrifuge again at 4,000 × g for 2 minutes. Discard S-Trap™ column.

24. Dry eluted peptides in a collection tube using a vacuum centrifuge at room temperature for 1 −2 hours, checking every half hour. Remove promptly when dry.

These peptides can now be used directly for MS analysis, but it is recommended to perform peptide quantification (Support Protocol 2) to advise on how much sample to load onto the MS. Alternatively, if additional depth of coverage is needed, peptides can be further fractionated using Support Protocol 3.

Starting with 50 mg human heart tissue, Basic Protocol 1 will typically yield sufficient peptide quantity for 30–40 injections of 1 μg peptide per injection onto a nanospray LC-MS system, with more than 4,000 unique proteins identified per injection (Fig. 3).

ALTERNATE PROTOCOL 1: PREPARATION OF LOW TO MODERATE COMPLEXITY PEPTIDE SAMPLES FOR MASS SPECTROMETRY ANALYSIS USING IN-SOLUTION PROCESSING

This protocol outlines the steps required to prepare low to moderate complexity samples for MS analysis, and describes how to solubilize proteins from RP-HPLC fractions, affinity purification fractions or lyophilized protein samples, or how to homogenize cells. Then, steps to reduce disulfide bonds and alkylate free cysteines, and enzymatically digest the protein are described (Fig. 4). At the end of Alternate Protocol 1, the user should proceed to Alternate Protocol 2 for sample cleanup. Subsequently, Support Protocol 3 can be optionally used to fractionate peptides based on their hydrophobicity for samples where enhanced sequence coverage of lower abundance peptides is required. Finally, Support Protocol 2 can be used directly after Alternate Protocol 2 or Support Protocol 3 to inform how much of the sample should be used for MS analysis, or to normalize total peptide in the case of quantitative analyses (Fig. 1C).

NOTE: Use only HPLC and/or LC-MS grade solvents throughout the protocol. All steps are performed at room temperature unless otherwise noted.

Materials:

Sample containing protein of interest.

This protocol is suitable for processing a wide range of sample types including purified proteins in lyophilized form, RP-HPLC and affinity chromatography fractions containing protein(s) of interest, and whole or fractionated lysates of cells. See Strategic Planning section for important notes, but 1–2500 μg protein is the suggested starting amount.

Purified water (18 MΩ)

1X Phosphate Buffered Saline (Gibco, PN 10010–023)

Glacial acetic acid (Thermo Fisher Scientific, PN BP1185)

1 N sodium hydroxide (NaOH) (10 N NaOH, Sigma Aldrich, SX0607N-6) (to adjust pH if needed)

100 mM aqueous ammonium bicarbonate (NH4HCO3, Sigma Aldrich, PN 09830) in LC-MS grade water (Fisher Scientific, PN W6), pH 8.5) (See Reagents and Solutions)

250 mM aqueous ammonium bicarbonate (See Reagents and Solutions)

Invitrosol™ LC-MS Protein Solubilizer Kit (Invitrogen, PN MS10007)

Acetonitrile (LC-MS grade, Thermo Fisher Scientific, PN A955)

Cell lysis buffer (See Reagents and Solutions)

100 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma, PN C4706) in LC-MS grade water

400 mM acrylamide (Sigma, PN A3553)

Trypsin/LysC enzyme combination (Promega, PN V5071)

1.0% (v/v) trifluoroacetic (TFA) (LC-MS grade, Fisher Scientific, PN A116) acid in LC-MS grade water (See Reagents and Solutions)

Narrow-range pH paper

20 mL disposable glass vials (for solution storage)

Low retention 1.5 microfuge tubes (Thermo Fisher Scientific, PN 02-681-320)

10 μL glass syringe or other glass pipette device

Variety of positive displacement pipettes (*e.g.* P10 – P1000)

Low retention pipette tips (*e.g.* 0.5 μL – 1 mL)

Mixing incubator (*e.g.* Thermomixer)

Vacuum centrifuge (*e.g.* SpeedVac)

Block sonicator (*e.g.* Hielscher, UP200St)

Laboratory sealing film (*e.g.* Parafilm)

Reagents and equipment for peptide and protein quantification (see Support Protocols 1 and 2)

Reagents and equipment for desalting/cleanup prior to LC-MS analysis (see Alternate Protocol 2)

Protocol Steps

Prepare the protein sample

1a. To prepare a sample from an RP-HPLC or affinity purification fraction: Dry the sample containing the proteins of interest via vacuum centrifugation. The evaporation should be performed at room temperature to avoid degradation of the sample; the amount of time required will be dependent on the volume and

organic content of the sample. Resuspend dried protein in 100 μL of 100 mM NH_4HCO_3, pH 8.5, with rigorous vortexing for 30 seconds. Spot an aliquot of the solution (1 μl) on narrow-range pH paper. Adjust to pH 8.0 with glacial acetic acid or 1 N sodium hydroxide as necessary.

The volume used for resuspension depends on the amount of protein in the original sample. Generally, a solution of 2 μg/μL works well for protein quantification and digestion. A volume of 100 μL would be appropriate for an unknown amount, but further dilution may be necessary depending on solubility (Strategic Planning).

The NH_4HCO_3 solution should be prepared fresh, as the pH of the solution will increase over time at room temperature. It is possible to store the NH_4HCO_3 solution for several days at 4°C, but the pH should be checked prior to use. The pH also makes it prone to bacterial growth, which can contaminate samples.

1b. To prepare a lyophilized protein sample: Resuspend dried protein (50–1000 μg, see note on concentration above) in 100 μL of 100 mM NH_4HCO_3, pH 8.5, with rigorous vortexing for 30 seconds. Spot an aliquot of the solution (1 μl) on narrow-range pH paper. Adjust to pH 8.0 with acetic acid or 1 N sodium hydroxide as necessary.

1c. To prepare a protein sample from cultured and counted cells:

- **i.** Count cells and collect by centrifugation (300 × g for 5 minutes at room temperature).
- **ii.** Aspirate and discard supernatant.
- **iii.** Resuspend in culture media based on cell count and add a minimum of 1 million cells and a maximum of 20 million cells to each sample or 1.5 mL tube (*e.g.* a minimum of 1 million cells in 250 μL and a maximum of 20 million cells in 800 μL).
- **iv.** Collect cells by centrifugation (300 × g for 5 minutes at room temperature).
- **v.** Aspirate and discard supernatant.
- **vi.** Add 1 mL of 1X PBS. Gently pipette up and down 3–5 times using a P1000 pipette until cells are well resuspended.
- **vii.** Collect cells by centrifugation (300 × g for 5 minutes at room temperature).
- **viii.** Aspirate and discard liquid.
- **ix.** Add 1 mL of 1X PBS. Gently pipette up and down 3–5 times using a P1000 pipette until cells are well resuspended.
- **x.** Collect cells by centrifugation (300 × g for 5 minutes at room temperature).

xi. Aspirate and discard liquid.

xii. Add cell lysis buffer according to cell count shown below. Mix by pipetting up and down 3–5 times. Keep on ice throughout the protocol.

Number of cells	1–2 million	5 million	10 million	15–20 million
Lysis buffer	250 µL	300 µL	500 µL	600–800 µL

If samples appear to have a large insoluble pellet after sonication in step xiii, add more volume in this step, up to a total maximum of 800 µL.

xiii. Place tube in the block sonicator's tube adaptor that is suspended above an ice bucket. Make sure that both the bottom and top of the tube are embedded in ice. Sonicate for 10 seconds on and then 10 seconds off, 3 times, at 30% power. Wait 1 minute and repeat the cycle two more times if ≥ 5 million cells are digested. Keep on ice afterwards.

These parameters are suitable for the Hielscher UP200St block sonicator. Optimize the power levels according to your sonicator. While a probe sonicator may be used, this increases the chance for cross-contamination as the probe is reused and requires careful cleaning between uses. The block sonicator used here enables samples in a closed tube to be processed without cross-contamination.

2. Perform protein quantification. See Support Protocol 1.

Determining the amount of protein in the sample will inform the amount of enzyme to use in later steps. This information can also be used to normalize among samples intended for quantitative analyses. As stated in the Strategic Planning section, for sample-limited cases, it is advisable to at least have an estimate of the total amount of protein based on a similar sample type, when possible. If, based on a representative sample, the amount of protein in the experimental sample is less than 10 µg, it will not be possible to perform protein quantification without using a majority of the sample for this step. However, if sufficient protein is indeed available, protein quantification is strongly advised.

Reduce disulfide bonds and alkylate free cysteine residues

5. Reduce disulfides by adding 100 mM of TCEP to the sample from Step 1 to a final concentration of 5 mM.

While reduction and alkylation (steps 3–5) are not required prior to enzymatic or chemical cleavage of proteins that do not contain cysteines, these steps will typically provide enhanced protein sequence coverage of

most proteins as they help unfold the protein and maintain it in an open configuration, thereby facilitating cleavage.

6. Cap tubes and place samples in a mixing incubator at 37°C for 30 minutes.

   Throughout this protocol, agitation can be achieved with end-over-end rotation or vortexing.

7. Alkylate cysteines by adding 400 mM acrylamide to a final concentration of 40 mM, cap tubes, and place samples in a mixing incubator at 37°C for 30 minutes.

Digest Protein

8. Add appropriate amount of Trypsin-LysC or other digestion enzyme (*e.g.* chymotrypsin, trypsin, LysC, or AspN) or chemical (*e.g.* cyanogen bromide/70% formic acid) based on total amount of protein present. This will vary among enzymes. If using trypsin, add sufficient enzyme for a final trypsin:protein ratio of between 1:20 to 1:100 (w/w).

   The amount of trypsin needed will vary depending on the amount of protein in the sample. While longer digestion times may lead to higher sequence coverage, this will also result in increased loss of some peptides, which can affect quantification. Shorter digestion times can benefit workflow speed but may result in lower sequence coverage.

   Many commercial vendors provide proteomics-grade trypsin in convenient aliquots (e.g. 1- to 25-μg vials, such as Promega, PN V5083), which eliminate the need to weigh out the enzyme. To minimize trypsin autolysis, which will add extra peaks to the MS spectra, use the minimum amount of trypsin. Typically, a ratio of 1:20 (w/w) is sufficient for complete digestion of 10 μg protein within 4 hours (e.g. 0.5 μg of enzyme). If protein concentration is especially high (e.g 2500 μg) or the accessibility of trypsin to the cleavage sites is hampered (e.g. due to insolubility), it may be helpful to add another aliquot of trypsin, perform a short digestion (4 hours) with LysC prior to the addition of trypsin, or use combination enzymes such as Trypsin-LysC (Promega, Trypsin/LysC PN V5071)).

   The activity of trypsin is enhanced in the presence of acetonitrile (10% to 50% v/v), and, thus, acetonitrile may be added to aid in the solubilization of the protein during tryptic digestion (Waas et al., 2014).

9. Use 1 μl of the sample to test the pH using a narrow-range pH paper. As trypsin activity is greatest at pH 8.0, adjust to pH 8.0 with acetic acid or 1 N sodium hydroxide as necessary.

10. Vortex briefly, cap and seal tube with Parafilm, and incubate in a mixing incubator at 37°C for 4 – 17 hours.

11. Proceed to Support Protocol 2 for peptide quantification, which will be used to then inform sample cleanup (Alternate Protocol 2), prior to MS analysis.

Alternatively, if additional depth of coverage is needed, peptides can be further fractionated using Support Protocol 3.

ALTERNATE PROTOCOL 2: DETERGENT, POLYMER, AND SALT REMOVAL FROM PEPTIDE SAMPLES PRIOR TO MASS SPECTROMETRY ANALYSIS USING SP2 PROCESSING

Detergents and salts are common ingredients in sample preparation buffers. Polymers (*e.g.* polyethylene glycol (PEG)) resulting from the manufacturing process of laboratory consumables (*e.g.* tubes, filters) can also contaminate proteomics samples. Detergents, polymers, and salts will interfere with chromatographic separation and ionization of peptides and must, therefore, be removed from the samples prior to MS analysis. While reversed-phase methods using filter cartridges or tips are popular for peptide desalting, they are not capable of removing detergents or PEG. The recently described SP2 method uses carboxylate-modified paramagnetic particles to remove detergents, PEG, and salts from peptide samples (Waas et al., 2019). This protocol describes how to perform the SP2 method for contaminant removal from peptide samples prior to MS analysis (Fig. 5). Peptides resulting from step 9 in Alternate Protocol 1, or alternative sample preparation strategies such as on-filter digestions, can be cleaned by this method.

NOTE: Use only HPLC and/or LC-MS–grade solvents throughout the protocol. All steps are performed at room temperature unless otherwise noted.

Materials:

Peptide sample from Alternate Protocol 1, step 9 (concentration and mass should be determined beforehand following Support Protocol 2)

Sera-Mag SpeedBeads Carboxylate-Modified Particles, 50 μg/μL (hydrophilic, Cytivia, 45152105050250)

Sera-Mag SpeedBeads Carboxylate-Modified Particles, 50 μg/μL (hydrophobic, Cytivia, 65152105050250)

Acetonitrile (LC-MS grade, Thermo Fisher Scientific, PN A955)

Water (LC-MS grade, Thermo Fisher Scientific, PN W6)

10% formic acid (FA) (FA, LC-MS grade, Fisher Scientific, PN A117) in LC-MS water (see Reagents and Solutions)

Elution solution (2% v/v LC-MS grade acetonitrile in LC-MS grade water)

Magnetic rack (*e.g.* Permagen, PN MSR24)

Mini centrifuge

Thermomixer or similar

Vortexer

Orbital shaker

20 mL disposable glass vials (for solution storage)

Low retention 1.5 microfuge tubes (Thermo Fisher Scientific, PN 02-681-320)

10 µL glass syringe or other glass pipette device

Variety of positive displacement pipettes (*e.g.* P10 – P1000)

Low retention pipette tips (*e.g.* 0.5 µL – 1 mL)

Gel-loading tips (200 µL)

Protocol Steps

Prepare Particles

1. Remove both sets of Sera-Mag SpeedBeads Carboxylate-Modified particle stocks (hydrophilic and hydrophobic) from 4°C storage.
2. Place particle stocks on an orbital shaker for 30 minutes to allow them to warm to room temperature and to yield a homogeneous suspension.
3. Shake and vortex sufficiently to fully suspend the particles prior to use.

   This may require prolonged and vigorous mixing.
4. Combine 100 µL of hydrophilic particles with 100 µL of hydrophobic particles into a low retention 1.5 mL microfuge tube, and add 800 µL of LC-MS grade water.
5. Place the microfuge tube on a magnetic rack and let the particles settle for 2 minutes.
6. Carefully remove the supernatant with a 200 µL gel-loading tip and discard supernatant and tip.
7. Remove the microfuge tube from the magnetic rack and rinse the particles with 1000 µL LC-MS grade water.
8. Mix thoroughly by pipetting up and down 3–5 times using a P1000 pipette.
9. Return the microfuge tube to the magnetic rack and let the particles settle for 2 minutes.
10. Carefully remove the supernatant with a 200 µL gel-loading tip and discard supernatant and tip.
11. Repeat steps 7–10 twice more.
12. Add 200 µL of LC-MS grade water to resuspend the particles at 50 µg/µL and store at 4°C.

    Prepared particles can be stored at 4°C for 6 months. Do not freeze the particles.

Cleanup Peptides

13. Remove prepared particles from step 12 from 4°C storage.

14. Vortex prepared particles prior to immediate use to ensure they are well suspended.

15. Obtain peptides resulting from step 9 in Alternate Protocol 1.

16. Add prepared particles corresponding to 20 times the mass of peptides to a low retention 1.5 mL microfuge tube containing peptides to be cleaned.

    Example: for 15 μg of peptide solution at 1 μg/μL, use 6 μL of particles. 6 μL of particles at 50 μg/μL equals 300 μg of particles, which is 20 times the mass of peptides.

17. Flick the microfuge tube containing the peptides and particle suspension 3–5 times to mix the sample.

18. Determine the amount of acetonitrile required to bring sample to a final concentration of 95% acetonitrile. The peptides in step 15 contain 20% acetonitrile. Therefore, use the following equation to determine amount of acetonitrile to be added:

$$Volume_{Acetonitrile} = \frac{(95\% \bullet Volume_{Particle}) + \left(\left(\%95 - \left[Acetonitrile_{Sample}\right]\right) \bullet Volume_{Sample}\right)}{5\%}$$

    Example: If digest contains 20% acetonitrile, the volume of particles used is 6 μL, and the volume of sample is 15 μL, add 339 μL acetonitrile:

$$Volume_{Acetonitrile} = \frac{(95\% \bullet 6\,\mu L) + ((95\% - [20\%]) \bullet 15\,\mu L)}{5\%} = 339\,\mu L$$

19. Add 100% acetonitrile to the microfuge tube so that the final acetonitrile concentration is 95%.

    Example: if digest contains 20% Acetonitrile, add 339 μL Acetonitrile

20. Mix by pipetting up and down 3–5 times using a P1000 pipette to ensure particles are well-dispersed, and then allow the mixture to settle for 2 minutes.

21. Place microfuge tube on magnetic rack for 1 minute.

22. Remove the supernatant with a 200 μL gel-loading tip, taking care not to disturb the particles. Discard the supernatant and tip.

23. Remove the microfuge tube from the magnetic rack and add 500 μL of 100% acetonitrile to cover the particles completely.

    At this step, particles may remain stuck on the side of the tube, so ensure the acetonitrile wash covers the surface area of the particles.

24. Repeat steps 20–22 once more.

25. Remove the microfuge tube from the magnetic rack.

26. Determine the elution volume, considering that the percent recovery is maximized when using 9 times the volume of particles.

27. Add desired volume of elution solution to particles.

    Example: if 6 μL of particles was added in step 16, add 54 μL (9 times the volume of particles).

28. Vortex the microfuge tube containing particles in elution solution on the side for 30 seconds, to remove the particles from the tube wall and to disperse them.

29. To collect any sample that may be on the wall of the tube, briefly centrifuge the samples for 3 seconds using a benchtop mini-centrifuge (*i.e.* place tubes in mini centrifuge, turn the centrifuge on, and then immediately turn it off).

30. Place the microfuge tube on the magnetic rack and allow the particles to settle for 1 minute.

31. Using a 200 μL gel-loading tip, remove the supernatant and transfer to a clean microfuge tube, taking care not to disturb the particles.

    The supernatant contains cleaned peptides.

32. Repeat steps 30–31 once more.

33. Add 10% FA to a final concentration of 0.1% FA.

34. Centrifuge sample at 14000 × *g* for 10 minutes and then transfer the supernatant to a sample vial for MS analysis.

    Starting with 5 million B lymphocytes, sample preparation by Alternate Protocol 1 followed by cleanup using Alternate Protocol 2 will typically yield enough peptide quantity for 50 injections of 1 μg peptide per injection onto a nanospray LC-MS system, with more than 4,000 unique proteins being discovered per injection (Fig 6).

SUPPORT PROTOCOL 1: PROTEIN QUANTIFICATION USING THE PIERCE™ 660 NM ASSAY

Protein quantification is an important step in many bottom-up proteomics workflows (including the ones described here), as it informs how much enzyme to use for optimal digestion efficiency and which size (binding capacity) S-Trap™ column to use. Several strategies are available for the determination of protein concentration (*e.g.* Bradford, bicinchoninic acid assay, NanoDrop™, and Qubit™), but they vary according to interferences allowed (*e.g.* SDS, Tween®20, Triton), sensitivity, and dynamic range. The Pierce™ 660nm Protein Assay is suitable for a range of proteomics applications and can be used to quantify samples that contain detergents like SDS and, hence, is compatible with workflows that require this detergent for enhancing protein solubilization (as is Basic Protocol 1). This protocol describes how to perform protein quantification using the Pierce™ 660nm Protein Assay.

Materials:

Sample from Alternate Protocol 1 step 2 or Basic Protocol 1 step 4

Pierce™ 660nm Protein Assay Kit (Pierce, PN 22662)

Pierce™ 660nm Protein Assay Reagent (Pierce, PN 22660)

Pre-diluted Protein Assay Standards

The Bovine Serum Albumin (BSA) Set contains standardized BSA solutions at a specific concentration, from 125 to 2000 µg/mL in 0.9% saline and 0.05% sodium azide. You will need two additional standards, 62.5 µg/mL and 31.25 µg/mL standard, which you need to prepare (see below).

Ionic Detergent Compatibility Reagent (IDCR, Pierce, PN 22663)

If using Pierce™ 660nm Protein Assay kit for S-Trap™ samples, the ionic detergent compatibility reagent is necessary for consistent readings.

Purified water (18 MΩ)

Multi-channel pipette

96-well flat bottom clear plate (Thermo Fisher Scientific, PN 12-565-501)

25 mL reservoir

Spectrophotometer (*e.g.* Varioskan LUX Multimode Microplate Reader)

Protocol Steps

Prepare Sample

1. Dilute samples before protein quantification. For this, add 5 µL of each sample to two tubes. Then, add 95 µL of purified water to one tube and label it DF=20 (*d*ilution *f*actor 20). Lastly, add 195 µL to the other tube and label it DF=40. Mix well.

   In our experience, these dilutions typically work well when starting with 5 million B cells for Alternate Protocol 1 or 50 mg heart tissue for Basic Protocol 1. If the sample is found to be outside the concentration range of the protein standards, dilute within the range and reanalyze.

2. Add 100 µL of purified water to a low retention 1.5 microfuge tube and then add 100 µL of the 125 µg/mL BSA standard. This will be the 62.5 µg/mL standard.

3. Add 100 µL purified water to a low retention 1.5 microfuge tube and then add 100 µL of the 62.5 µg/mL standard. This will be the 31.25 µg/mL standard.

4. Add 10 µL of each standard, unknown sample, and the appropriate blank water sample into their respective wells of a 96-well microplate well.

   Standards, blanks, and samples should be added in duplicates.

5. Add 20 mL of Pierce™ 660nm Protein Assay Reagent to a 50 mL conical tube and add the contents of an Ionic Detergent Compatibility Reagent (IDCR) packet, according to the manufacturer’s instructions. Cap and mix well to dissolve.

IDCR is an additive to the assay reagent that improves the sensitivity and reproducibility of the assay in the presence of SDS.

6. Add 150 µL of the IDCR Protein Assay Reagent to each well, for a final well volume of ~160 µL.
7. Mix plate by gently tapping palm against the side to the plate three times.
8. Incubate plate for 5 minutes in the dark.

Perform Measurement and Calculations

9. Measure absorbance at 660 nm using a spectrophotometer.
10. Determine concentration. Depending on the spectrophotometer used, the concentration including the dilution factor may be calculated for you. In this case, the measurement will result in a unit of µg/mL. To convert to total µg/µL divide the concentration by 1000. To calculate the total µg in your sample, multiply that number by the volume remaining in the sample.

    Table 1 shows some typical concentration results obtained from various starting samples.

SUPPORT PROTOCOL 2: PEPTIDE QUANTIFICATION – PIERCE QUANTITATIVE FLOROMETRIC PEPTIDE ASSAY

Peptide quantification is an important step for evaluating yield of the processing steps used to generate peptides from proteins and for normalizing the total peptide amount analyzed in quantitative workflows. Peptide quantification can be done immediately prior to MS to inform whether the sample should be diluted prior to analysis. It can also be performed prior to Alternate Protocol 2 and Support Protocol 3 to inform mass of peptides to use for each application. This protocol describes how to perform peptide quantification using the Pierce™ Quantitative Fluorometric Peptide Assay.

Materials:

Sample resulting from Alternate Protocol 1 step 9 or Basic Protocol 1 step 23.

Pierce™ Quantitative Fluorometric Peptide Assay (do not premix items)

- Fluorometric Peptide Assay Buffer
- Fluorometric Peptide Assay Reagent
- Peptide Digest Assay Standard (1 mg/mL)

Purified Water

Multi-channel pipette

96-well flat bottom black plate (Thermo Fisher Scientific, PN 12-566-07)

25 mL reservoir

Spectrophotometer (*e.g.* Varioskan LUX Multimode Microplate Reader)

Protocol Steps

Prepare Sample

1. Use the information below to prepare a dilution series of the Peptide Digest Assay Standard in separate tubes. Use purified water as diluent.

The volumes below will be enough for one set of measurements where each standard is placed into two wells of a 96-well plate and used as reference for all experimental samples in the plate. If the user is quantifying more samples than can fit into a single 96-well plate, then additional standards should be prepared so that each plate will contain standards in duplicate. Make standards fresh before use.

Tubes	Volume of Diluent, µL	Volume of Standard, µL	Final Standard Concentration, µg/mL
A	0	50 of stock	1000
B	25	25 of Tube A dilution	500
C	25	25 of Tube B dilution	250
D	25	25 of Tube C dilution	125
E	25	25 of Tube D dilution	62.5
F	25	25 of Tube E dilution	31.3
G	25	25 of Tube F dilution	15.3
H	25	25 of Tube G dilution	7.8
Blank	20	0	0

2. Dilute samples before quantification by adding 5 µL of each sample to two tubes. Add 20 µL of purified water to one tube and label it DF=5. Add 45 µL to the other tube and label it DF=10. Mix well.

These dilutions typically work well when starting with 5 million B cells for Alternate Protocol 1 or 50 mg heart tissue for Basic Protocol 1. If the sample is found to be outside the concentration range of the peptide standards, dilute within the range and reanalyze.

11. Add 10 µL of each standard, unknown sample, and the appropriate blank sample into a microplate well.

Standards, blanks, and samples should be aliquoted in duplicates.

3. Add 70 µL Fluorometric Peptide Assay Buffer to each well.

4. Add 20 µL Fluorometric Peptide Assay Reagent to each well.

5. Mix plate by gently tapping palm against the side to the plate three times.

6. Incubate plate for 5 minutes in the dark.

Perform Measurement and Calculations

8. Measure absorbance using wavelength excitation at 390 nm and emission of 475 nm using a spectrophotometer.

9. Determine concentration. Depending on the spectrophotometer used, the concentration including the dilution factor may be calculated for you. In this case, the measurement will result in a unit of µg/mL. To convert to total µg/µL, divide the concentration by 1000. To calculate the total µg in your sample, multiply that number by the volume remaining in the sample.

SUPPORT PROTOCOL 3: HIGH PH FRACTIONATION OF COMPLEX PEPTIDE SAMPLES

This protocol describes how to perform peptide fractionation using high pH reversed-phase peptide fractionation spin columns (Fig. 7). Fractionation of a single peptide mixture by this protocol will generate ten lower-complexity samples that can be analyzed separately by MS. In doing so, greater depth of proteome coverage can be achieved for moderate to high complexity samples where the dynamic range of protein abundances makes it more challenging to detect low abundance peptides. This protocol can be used directly after Basic Protocol 1 and Alternate Protocol 1, or other preparation strategies. The sample is loaded onto a washed and equilibrated spin column and peptides are eluted with increasing amounts of acetonitrile, at a high pH. Solvent is evaporated and peptides are then quantified before MS analysis.

NOTE: Use only HPLC and/or LC-MS grade solvents throughout the protocol. All steps are performed at room temperature unless otherwise noted.

Materials:

Sample from Alternate Protocol 1 that has been cleaned using Alternate Protocol 2 step 34, or Sample from Basic Protocol 1, step 23 (maximum 100 µg).

High pH Reversed-Phase Peptide Fractionation Kit (Pierce, PN 84868)

0.1% triethylamine (TEA) (included in kit)

1% trifluoroacetic acid (TFA) (TFA, Sigma Aldrich, PN 28904) in LC-MS grade water (Fisher Scientific, PN W6)

Acetonitrile (LC-MS grade, Fisher Scientific, PN A955)

Water (LC-MS grade, Fisher Scientific, PN W6)

5% v/v formic acid (FA) (FA, LC-MS grade, Fisher Scientific, PN A117) in 95% v/v LC-MS grade water (see Reagents and Solutions)

5% v/v formic acid (FA) (FA, LC-MS grade, Fisher Scientific, PN A117) in 95% v/v LC-MS grade acetonitrile (see Reagents and Solutions)

0.1% v/v formic acid (FA) (FA, LC-MS grade, Fisher Scientific, PN A117) in 2% v/v LC-MS grade acetonitrile in LC-MS grade water (see Reagents and Solutions)

Low retention 2.0 microfuge tubes (Fisher Scientific, PN 02-681-321)

Microcentrifuge with adjustable rotor speed up to 7,000 × g

Vacuum centrifuge

Protocol Steps

Prepare the Elution Solvents

1. Prepare solutions in 2.0 mL microfuge tubes according to the information below. This provides sufficient volume for one sample. If fractionating more than one sample, scale accordingly.

Fraction #	Acetonitrile %	100 % Acetonitrile (µL)	0.1% TEA (µL)
1	5	15	285
2	7.5	22.5	277.5
3	10	30	270
4	12.5	37.5	262.5
5	15	45	255
6	17.5	52.5	247.5
7	20	60	340
8	50	150	150
Fraction #	**Acetonitrile %**	**Acetonitrile with 5% FA (µL)**	**5% FA (µL)**
9	15	45	255
10	50	150	150

Condition the Spin Columns

Do not exceed recommended centrifugation speeds. All steps can be performed at room temperature unless otherwise noted.

2. Remove the protective white tip from the bottom of the high pH fractionation spin column and discard. Place the column into a 2.0 mL microfuge tube.
3. Centrifuge at 5000 × g for 2 minutes to remove the solution and pack the resin material. Discard the liquid.
4. Wash the spin column by removing the top screw cap and adding 300 µL of 100% acetonitrile onto the column. Cap and centrifuge at 5000 × g for 2 minutes. Discard acetonitrile wash.

   Replace the cap during each centrifuge step to ensure that the spin column will not dry out, which could affect the final results.
5. Repeat step 4 once more.
6. Wash the spin column by removing the top screw cap and adding 300 µL of 0.1% TFA solution into the column. Cap and centrifuge at 5000 × g for 2 minutes. Discard 0.1% TFA wash.
7. Repeat step 6 once more.

Fractionate the Peptide Mixture

8. If using peptides from Alternate Protocol 1 that were cleaned with Alternate Protocol 2, dry them in a low retention 1.5 mL microfuge tube using a vacuum centrifuge set at room temperature for 1–2 hours. Check every 30 minutes and remove promptly once dry. If using dried peptides from Basic Protocol 1, proceed to step 9.

9. Dissolve all of the peptide sample in 300 μL of 0.1% TFA solution.

The maximum amount of peptide that can be loaded onto the high pH fractionation column is 100 μg. If following Basic Protocol 1 and Alternate Protocol 1 as described above, the total amount of peptide in the resulting samples will be less than 100 μg. Therefore, the entire sample can be loaded onto the fractionation column. If using other than the recommended starting material or amounts described for Basic Protocol 1 and Alternate Protocol 1, perform peptide quantification following Support Protocol 2 and use a maximum of 100 μg for this step.

10. Place the spin column into a new 2.0 mL microfuge tube.

11. Add the 300 μL of sample solution from step 9 into the top of the column, cap, and centrifuge at 3000 × g for 2 minutes. Retain eluate as “flow-through” fraction for troubleshooting only.

As with most loading and washing steps, it is necessary to retain flow through solutions in case peptides were not retained on the packing material. If there are no peptides in the samples, this solution can be tested during troubleshooting.

12. Place the column into a new 2.0 mL microfuge tube.

13. Add 300 μL of water into the top of the column, cap, and centrifuge at 3000 × g for 2 minutes. Retain wash for troubleshooting only.

As with most loading and washing steps, it is necessary to retain flow through solutions in case peptides were not retained on the packing material. If there are no peptides in the samples, this solution can be tested during troubleshooting.

14. Place the column into a new 2.0 mL microfuge tube labeled “Fraction 1”.

15. Add 300 μL of the appropriate elution solution described in step 1 (*e.g.* 5% acetonitrile, 0.1% TEA) and centrifuge at 3000 × g for 2 minutes to collect the fraction. Retain eluate as “fraction #1”.

The first elution solution is fraction #1. A total of 10 elution solutions (prepared according to the table in step 1) will be added to the same column in 10 steps.

16. Repeat Step 14 and 15 for the remaining step gradient fractions using the appropriate elution solutions and a new microfuge tube each time (*i.e.* elution solutions for fractions #2–10).

17. Evaporate eluted peptides in a low retention 2.0 mL microfuge tube using a vacuum centrifuge set at room temperature for 1–2 hours. Check every 30 minutes and remove promptly once dry. The resulting peptide samples can then be used for MS analysis.

    Starting with 50 μg of peptides resulting from applying Alternate Protocol 1 to human ventricle heart tissue, the high pH fractionation method in Support Protocol 3 will typically yield sufficient peptide quantity for 3 injections of 1 μg peptide per injection per fraction onto a nanospray LC-MS system. In total, more than 7,000 unique protein identifications per set of fractions can typically be identified (Fig. 8).

REAGENTS AND SOLUTIONS:

100 mM aqueous ammonium bicarbonate (NH_4HCO_3), pH 8.5 (25 mL)

1. Weigh 198 mg of ammonium bicarbonate and add to 25 mL of LC-MS grade water. Mix well.
2. Adjust pH to 8.5 with glacial acetic acid.

250 mM aqueous ammonium bicarbonate (NH_4HCO_3), pH 8.5 (10 mL)

1. Weigh 198 mg of ammonium bicarbonate and add to 10 mL of LC-MS grade water. Mix well.
2. Adjust pH to 8.5 with glacial acetic acid.

Cell lysis buffer (40% invitrosol, 100 mM ammonium bicarbonate, 40% acetonitrile)

1. Prepare cell lysis buffer for Alternate Protocol 1 according to the number of cells to be used, as described below. For example, if using 5 million cells, add 300 μL of lysis buffer.

Number of cells	1–2 million	5 million	10 million	15–20 million
Lysis buffer	250 μL	300 μL	500 μL	600–800 μL

2. To prepare 250 μL of lysis buffer, mix 100 μL of invitrosol, 100 μL of acetonitrile, and 50 μL of 250 mM ammonium bicarbonate.
3. Make buffer fresh before use and keep on ice.

S-Trap™ binding/wash buffer (90% methanol, 100 mM TEAB), pH 7.1 (10 mL)

1. Add 90 mL of HPLC grade methanol to 10 mL of 1M TEAB and mix well.
2. Adjust pH to 7.1 with phosphoric acid (~0.1 mL).
3. Store at room temperature for one month.

12% Phosphoric Acid (10 mL)

1. Add 1.2 mL of 85% phosphoric acid to 8.8 mL of MilliQ water. Mix well.
2. Store at room temperature.

SDS Tissue Lysis buffer (5% SDS, 50mM TEAB), pH 7.55 (10 mL)

1. Add 5 mL of 10% SDS buffer to 4.5 mL of purified water and 0.5 mL of 1M TEAB. Mix well.
2. Adjust pH to 7.55 with 12% phosphoric acid (~20 µL).
3. Store at room temperature for up to one day.

SDS Cell Lysis buffer (5% SDS, 50mM TEAB), pH 7.55 (10 mL)

1. Add 5 mL of 10% SDS buffer, 0.02 mL of 1M Magnesium Chloride, and 0.5 mL of 1M TEAB, to 4.5 mL of purified water. Mix well.
2. Adjust pH to 7.55 with 12% phosphoric acid (~20 µL).
3. Store at room temperature for up to one day.

10% formic acid in LC-MS water (10 mL)

1. In a glass scintillation vial, add 1.0 mL of 100% formic acid to 9 mL of LC-MS grade water using a glass syringe dedicated to acid or other disposable glass pipette (Do not use plastic to avoid plastic leaching contamination).
2. Mix well and store at room temperature for up to one month.

0.2% formic acid in LC-MS water (5 mL)

1. Add 0.1 mL of 10% formic acid to 4.9 mL of LC-MS grade water in a glass scintillation vial.
2. Mix well and store at room temperature for up to two weeks.

0.2% formic acid in 50% LC-MS grade acetonitrile (5 mL)

1. Add 2.5 mL of LC-MS grade acetonitrile to 2.4 mL of LC-MS grade water in a glass scintillation vial.
2. Add 0.1 mL of 10% formic acid.
3. Mix well and store at room temperature for up to two weeks.

0.1% formic acid in 2% LC-MS grade acetonitrile (5 mL)

1. Add 0.1 mL of LC-MS grade acetonitrile to 4.85 mL of LC-MS grade water in a glass scintillation vial.
2. Add 0.05 mL of 10% formic acid.
3. Mix well and store at room temperature for up to two weeks

5% formic acid in LC-MS grade acetonitrile (5 mL)

1. Add 4.75 mL of LC-MS grade acetonitrile to a glass scintillation vial.
2. Add 0.25 mL of 100% formic acid using a glass syringe dedicated to acid or other disposable glass pipette (Do not use plastic to avoid plastic leaching contamination).
3. Mix well and store at room temperature for up to two weeks

5% formic acid in LC-MS grade water (5 mL)

1. Add 4.75 mL of LC-MS grade water to a glass scintillation vial.
2. Add 0.25 mL of 100% formic acid using a glass syringe dedicated to acid or other disposable glass pipette (Do not use plastic to avoid plastic leaching contamination).
3. Mix well and store at room temperature for up to two weeks

10% trifluoroacetic acid in LC-MS water (10 mL)

1. In a glass scintillation vial, add 1.0 mL of 100% trifluoroacetic acid to 9 mL of LC-MS grade water using a glass syringe dedicated to acid or other disposable glass pipette (Do not use plastic to avoid plastic leaching contamination).
2. Mix well and store at room temperature for up to one month.

1% trifluoroacetic acid in LC-MS grade water (5 mL)

1. Add 4.5 mL of LC-MS grade water in a glass scintillation vial.
2. Add 0.5 mL of 10% trifluoroacetic acid.
3. Mix well and store at room temperature for up to two weeks.

1% trifluoroacetic acid in 80% LC-MS grade acetonitrile (5 mL)

1. Add 4.0 mL of LC-MS grade acetonitrile to 0.5 mL of LC-MS grade water in a glass scintillation vial.
2. Add 0.5 mL of 10% trifluoroacetic acid.
3. Mix well and store at room temperature for up to two weeks.

COMMENTARY

BACKGROUND INFORMATION:

Mass spectrometry (MS) is an essential analytical technique in modern biological and physical science research, where it plays a principal role in protein identification, characterization, and quantification (Aebersold and Mann, 2003, 2016; Biemann, 2014). Its popularity for the analysis of proteins is due to multiple reasons. For instance, unlike gel-electrophoresis, which separates molecules according to their relative molecular mass (M_r) and can be affected by molecular shape, polarity, temperature, electric field, and solvent, MS

is dependent on the intrinsic property of a protein (*i.e.* mass). MS measures the mass-to-charge ratio of ions and is independent of many of the variables that affect size determination by electrophoresis. In addition, while antibody-based detection methods (*e.g.* flow cytometry, immunocytochemistry, western blot) are dependent on antibody specificity, which is itself dependent on protein isoform, post-translational modifications, and secondary/tertiary structure, MS can identify and characterize proteins despite these variables. Finally, MS requires no *a priori* knowledge of proteome composition to identity thousands of proteins from a single sample.

MS-based methods for proteomics can generally be categorized as top-down or bottom-up. Overall, bottom-up and top-down approaches are often complementary as they provide different levels of information, and each has their own technical challenges and advantages. Top-down methods preserve the stoichiometry among post-translational modifications, proteolytic cleavage products, and products of splicing events, among others (Kelleher et al., 1999). Despite these advantages, bottom-up methods remain popular for peptide identification, quantification, identification of interaction partners, and site-localization of post-translational modifications (Roux et al., 2012; Rhee et al., 2013; Gillet et al., 2016; Mayne et al., 2016; Kho et al., 2004; Sprung et al., 2005; Abello et al., 2010; Wollscheid et al., 2009; Frei et al., 2013; Sabarth et al., 2002). The popularity of bottom-up methods is due, in part, to their ease of implementation and available instrumentation and bioinformatic tools suitable for many different experimental workflows (Eng et al., 2013; Gluck et al., 2013; Wenger and Coon, 2013; Dorfer et al., 2014; Kim and Pevzner, 2014; Risk et al., 2013; Cox and Mann, 2008; Perkins et al., 1999; Eng et al., 1994).

Every sample preparation approach is biased and may not be suitable for all proteins. In other words, there is no universal bottom-up sample preparation approach that allows for simultaneous identification and characterization of high and low abundance proteins, soluble and membrane-bound, unmodified and modified, and secreted and localized proteins. For example, there are more than 200 known post-translational modifications, and identifying the proteins that carry these modifications requires specialized approaches to enrich specifically for these modifications in such a way that typically excludes the detection of other protein classes. The protocols described here, for instance, are suited to the identification and quantification of peptides and proteins, but generally not to the identification of post-translational modifications unless they are combined with enrichment methods specific to each modification type.

Importantly, all proteomics methods will be biased towards identifying proteins that are most soluble or abundant under the experimental conditions used. For example, Alternate Protocol 1 will be biased towards soluble, abundant proteins and will provide limited coverage of plasma membrane proteins compared to specialized approaches that are specifically designed to solubilize and enrich for this class of hydrophobic and low abundance proteins (Wollscheid et al., 2009; Kim et al., 2011; Shin et al., 2003). Moreover, Alternate Protocol 1 is unlikely to provide suitable proteome coverage of tissue due to high molecular heterogeneity, limited solubility of membrane and extracellular matrix proteins, and large dynamic range of protein concentrations present in such samples. When analyzing tissue or cells that are difficult to disrupt (*e.g.* muscle), the use of harsh detergents coupled

with rigorous physical disruption methods are typically necessary to enhance coverage of the proteome. However, standard laboratory detergents (*e.g.* SDS, Triton™X-100, Tween®20, CHAPS) are incompatible with MS. While MS-compatible surfactants are now commercially available (*e.g.* Invitrosol™, ProteaseMax™, RapiGest™ SF), they commonly need to be paired with chaotropic agents or organic solvents for maximum solubility of the proteome (Waas et al., 2014). The recent development of suspension trapping sample preparation (Zougman et al., 2014), Strap or S-Trap™, enables streamlined processing of samples that have been historically more challenging to work with because it achieves enzymatic digestion of proteins solubilized in SDS in a way that yields MS-compatible peptides that do not require further cleanup.

It is expected that the facile preparation methods described in this article will be easy to implement, with high success rates in laboratories with little to no experience in proteomics. However, alternative approaches can be considered. For Basic Protocol 1, alternatives to the S-Trap™ method for streamlined protein immobilization, digestion, and cleanup include spin filter-aided sample preparation (Manza et al., 2005; Wiśniewski et al., 2009), although another study found that S-Trap™ provides superior performance (Kr et al., 2018). The PreOmics iST Sample preparation kit and Thermo Scientific™ EasyPep™ are also designed for easy use, although we have not directly compared performance with the S-Trap™. SP3 is another strategy that uses carboxylate coated magnetic beads to achieve these three steps but may be more challenging to implement for novice users (Waas et al., 2019; Sielaff et al., 2017; Moggridge et al., 2018).

Relevant to Alternate Protocol 2, the SP2 protocol will remove many common contaminants from peptide samples, including salts, detergents (*e.g.* SDS, Tween®20, Triton), and PEG (Fig. 9), which overall improves chromatography and reduces time and costs associated with having to clean LC-MS systems after contaminations have been introduced. Overall, SP2 cleanup allows for higher sample throughput, improves instrument performance, and results in less downtime due to system maintenance compared to other cleanup methods (*e.g.* C_{18}) (Waas et al., 2019).

Low abundance proteins, such as cytokines, play important roles in many physiological processes (Hirano et al., 1990; Kitagawa and Saito, 1988). Furthermore, some biological processes are regulated by subtle changes in abundance or post-translational modifications of high and low abundance proteins (Andelova et al., 2020; Cundiff et al., 2016; Karve and Cheema, 2011). Therefore, the ability to dig deep into the proteome and detect both low and high abundance proteins is critical for many study designs. Several strategies are available for protein and peptide-level separation, which aim to spread the highly complex proteome across multiple fractions, creating multiple lower-complexity samples from a single high complexity sample. While LC-MS methods typically include on-line, low pH reversed-phase chromatography to separate complex peptide mixtures, there is a limit to the amount of separation achieved with typical gradients and column dimensions. For this reason, several multi-dimensional fractionation methods have been developed to further separate protein and peptide mixtures prior to MS analysis, including methods that use size exclusion chromatography (SEC) or hydrophilic interaction chromatography (HILIC) (Opiteck et al., 1998; Schirmer et al., 2003; Boersema et al., 2007). Variations of these approaches can be

performed using HPLC or spin-column based tips and filter tubes. For the off-line formats of SEC and HILIC, some form of desalting or cleanup is often required prior to MS analysis. In another off-line approach that is the basis of Support Protocol 3, high pH reversed-phase fractionation provides separation that is orthogonal to low pH reversed-phased separation and yields peptides that are in a solution that is directly compatible with MS analysis without requiring any further peptide cleanup or desalting.

CRITICAL PARAMETERS:

Generally, the ability to detect multiple peptides per protein contributes to higher confidence data regarding the identification and abundance of the protein. As such, the degree to which the protein is digested into peptides is a major contributor to the success —or lack thereof— in a bottom-up proteomics experiment. This is not to say that identifications based on a single peptide sequence are universally unacceptable, but that for general proteomics workflows aimed to simultaneously discover and quantify many proteins in a single experiment, a higher number of peptides generated and detected by MS will yield more protein identifications and more quantifiable data. Consequently, a key principle in bottom-up proteomics workflows is that the enzyme or chemical used for digestion must have access to the cleavage sites within the protein, and the solution must be at an appropriate pH for enzyme or chemical activity. The three-dimensional conformation of a protein in solution will affect accessibility to cleavage sites. Therefore, experimental conditions that enhance protein solubilization and unfolding —and thus promote enzymatic activity— will overall lead to maximum efficiency of cleavage (at least 85% of peptides with zero missed cleavages), yield more peptides, and result in better quality data. For this reason, users may consider sample-specific optimization of the steps described in Basic Protocol 1 and Alternative Protocol 1 to determine which conditions lead to the greatest amount of sequence coverage and the most reproducible detection of peptides of interest. An overview of common optimization steps is outlined in Fig. 10.

Reduction and alkylation of thiols to cleave disulfide bonds and prevent their reformation is a strategy that commonly leads to higher protein sequence coverage. Reduction of disulfide bonds can be achieved with either dithiothreitol (DTT) or TCEP. Compared to DTT, which works optimally at pH 7 to 9, TCEP is typically more efficient, works at a wider pH range (pH 2 to 11), and has no offensive odor. Therefore, our protocols use TCEP. Iodoacetamide (IAA) and acrylamide are both popular choices for alkylating reagents. Although we have found them to be interchangeable for the in-solution processing of lower complexity mixtures, acrylamide provides a slight advantage for reducing missed cleavages and increasing the number of protein identifications for very complex mixtures when using Basic Protocol 1 (Fig. 11). Importantly, when reduction and alkylation are performed, the alkylation results in modification of the cysteine residue (carbamidomethyl for IAA, propionamide for acrylamide), and this modification must be considered when performing MS data analysis. The concentrations and incubation times for DTT, TCEP, acrylamide, and IAA may vary depending on the amount of protein in the sample. In our experience, the concentrations and reduction/alkylation times provided in these protocols work well for the sample types we use most commonly (*e.g.* B lymphocytes, HeLa cells, human heart tissue), but concentrations of 5 to 10 mM for DTT and TCEP and 10 to 50 mM for IAA may also be

tested. Incubation times may range from 5 minutes to 1 hour for the reduction step, and 10 minutes to 1 hour for the alkylation step. Similarly, reduction can be performed at room temperature, 37°C, or 56°C.

Another key principle is that the enzyme or chemical selected will determine which peptide sequences are available for analysis. Only a fraction of the peptides resulting from digestion will be of a suitable mass-to-charge ratio (*m/z)* for MS/MS analysis. Therefore, any prerequisites for coverage of a specific site or region within a selected protein or proteins must be considered when planning the experimental design to ensure that the appropriate digestion enzyme or chemical is used.

For Alternate Protocol 1, one of the most important factors that can affect the efficiency of a trypsin digestion is pH. Users should be aware that some trypsin products come as lyophilized powder and others come in solution format. Typically, trypsin is dissolved in acidic conditions. Another important consideration is the solubility of the protein and the access of trypsin to the digestion sites within the protein sequence. Standard laboratory detergents (*e.g.* SDS, CHAPS, Triton, Tween®20) can suppress trypsin activity. Therefore, for in-solution processing, the MS-compatible surfactant Invitrosol (Invitrogen) and acetonitrile are added to increase the effectiveness of the enzymatic digestion (Waas et al., 2014). Alternative MS-compatible surfactants include Rapigest (Waters), PPS (ProteinDiscovery), MaSDeS (Chang et al., 2015), and Azo (Brown et al., 2019).

For Basic Protocol 1, when using tissue as a starting material, the choice of homogenization and subsequent sonication methods are critical for success. We discourage the use of a mortar and pestle, which results in incomplete homogenization. Multi-sample benchtop homogenizers (*e.g.* Barocycler (Pressure BioSciences), Bead Beater (MP Biomedical), BeadBlaster (Benchmark)) are available at a range of prices and capabilities, and in our experience, the bead-based homogenization method works well for disrupting human heart tissue. Equally important, the sonication step is critical for lysing the cells and shearing DNA. We discourage the use of probe sonicators, as using the same item to contact multiple samples can result in cross-contamination. Thus, we use a block sonicator which allows individual samples to be sonicated within enclosed microfuge tubes. If homogenization or sonication are not optimized, the S-trap™ may become clogged. Similar to Alternate Protocol 1, it is important to optimize digestion time and temperature for the sample and enzyme choice in Basic Protocol 1 by inspecting the sequence coverage and rate of missed cleavages in the search results. For example, using Basic Protocol 1, we find that a 2-hour trypsin/lysC digestion works well for heart tissue, but other cell types (e.g. B lymphocytes) can work well with trypsin for only 1 hour at 47°C. Finally, the maximum volume that can be loaded at one time onto the S-trap™ micro spin column is 300 µl (25 µl sample + 275 µl digestion buffer and other components). Therefore, while it is possible to begin with protein in a volume larger than 25 µl, this would require the user to use various 300 µl aliquots and perform multiple centrifugation steps to process the entire sample. S-trap™ columns, however, are available in various sizes (*e.g.* micro (≤ 100 µg protein), mini (100–300 µg protein), and midi (≥ 300 µg protein)) and if it is desired to process a larger amount of protein, Basic Protocol 1 can be adjusted according to the manufacturer's guidelines to facilitate the use of mini or midi S-trap™ columns.

The magnetic particles used in Alternate Protocol 2 settle during storage and form a mass that can be difficult to dissociate. A homogeneous mixture can be obtained when a combination of shaking (*e.g.* placing the particle stock on an orbital shaker for 30 minutes) and vortexing is used. It is recommended to prepare a stock of particles prior to sample cleanup and store them at 4°C, for up to 6 months. In our experience, the Sera-Mag SpeedBeads as well as the original Sera-Mag versions perform equivalently in terms of recovery and cleaning capacity. Whereas the SpeedBeads settle within fifteen seconds when placed on a magnetic rack, either type of particle is fully settled against the magnet after one minute. If using a low retention 1.5 mL microfuge tube, the total volume of sample + diluent + particle can be 75 µL. Maintaining the total volume at ≤ 50µL facilitates sample handling, as the addition of sufficient acetonitrile to achieve 95% can be performed in one step. Alternatively, SP2 cleanup with larger volumes can be performed in a 15 mL tube using a 15 mL tube magnetic rack.

For Support Protocols 1 and 2, accurate pipetting is essential. The user should follow best practices and check the accuracy of their pipettes and skill set via weight determination. Standards and blanks should be performed in duplicate and samples should be processed with two different dilutions in duplicate.

For Support Protocol 3, the percent acetonitrile can be changed to spread the distribution of peptides across fractions. Fractions may be combined to reduce MS instrument time needed to analyze the sample if their elution profile indicates they are complementary (*e.g.* fraction #1 would contain mostly hydrophilic peptides, while later fractions may not and would not elute at the same time with reverse phase chromatography).

The peptide samples resulting from Basic Protocol 1 and Alternate Protocol 1 are suitable for MS coupled to either liquid chromatography (LC) or matrix-assisted laser desorption/ ionization (MALDI) interfaces. Based on peptide quantification (measured or estimated), the samples can be used directly or diluted to an appropriate concentration prior to placing them in the sample vial for MS analysis. If using LC-MS, samples are typically diluted in aqueous mobile phase (or 2% acetonitrile in aqueous 0.1% FA) and we typically inject no more than 1 µg per replicate. However, the appropriate peptide concentration will depend on many factors, including instrumentation, method, column, sample complexity, and experimental goals. For Basic Protocol 1 and Alternate Protocol 1, the performance of the method will ultimately be assessed based on MS results. Although we report results that are typical of our experience, MS results (*e.g.* number of peptides and proteins identified) will be directly affected by the condition and type of the column used in the case of LC-MS analyses, the type and capabilities of the MS instrumentation, data acquisition settings, and data analysis details, as detailed previously (Han et al., 2008; Dave et al., 2011; Zhang et al., 2014). Therefore, it is possible that another user could apply the protocols expertly to the same sample types as described here but identify a different number of proteins because the MS instrument is configured slightly differently. Ultimately, results will depend on performance of the instrumentation and software used to analyze the data. Consequently, users are strongly encouraged to consult with the MS instrumentation operator before beginning an experiment, to ensure the experimental design is appropriate and that the peptide amount and sample volume are adequate for the instrumentation that will be used for analysis.

TROUBLESHOOTING:

In Table 2, we provide a list of common problems associated with each of the protocols described, and their solutions.

UNDERSTANDING RESULTS:

Examples of data we obtain from application of these methods are provided within each Protocol. In general, sample-specific optimization of each step of the workflows illustrated in Figs. 1 and 10 will result in increased digestion efficiency, sequence coverage, and proteome coverage. Examining the total number of proteins identified, number of peptides per protein, sequence coverage, enzyme specificity (*e.g.* full versus semi-tryptic peptides), and missed cleavages can inform whether the method requires optimization. High pH fractionation should increase the number of identified peptides compared to a non-fractionated sample.

TIME CONSIDERATIONS:

The protocols described here can be completed as follows:

Basic Protocol 1: One person can typically prepare a maximum of 18 samples in 8 hours, assuming the centrifuge used can hold this number of microfuge tubes simultaneously.

Alternate Protocol 1: One person can typically prepare a maximum of 18 samples in 12 hours, assuming the centrifuge used can hold this number of microfuge tubes simultaneously.

Alternate Protocol 2: One person can prepare 8–12 samples in 30 minutes assuming the magnetic rack can hold this number of microfuge tubes simultaneously.

Support Protocol 1: One person can typically prepare 6–8 samples in 30 min and 12 samples in 1 hour.

Support Protocol 2: One person can typically prepare 6–8 samples in 30 min and 12 samples in 1 hour.

Support Protocol 3: One person can typically prepare 3–6 samples in 3 hours.

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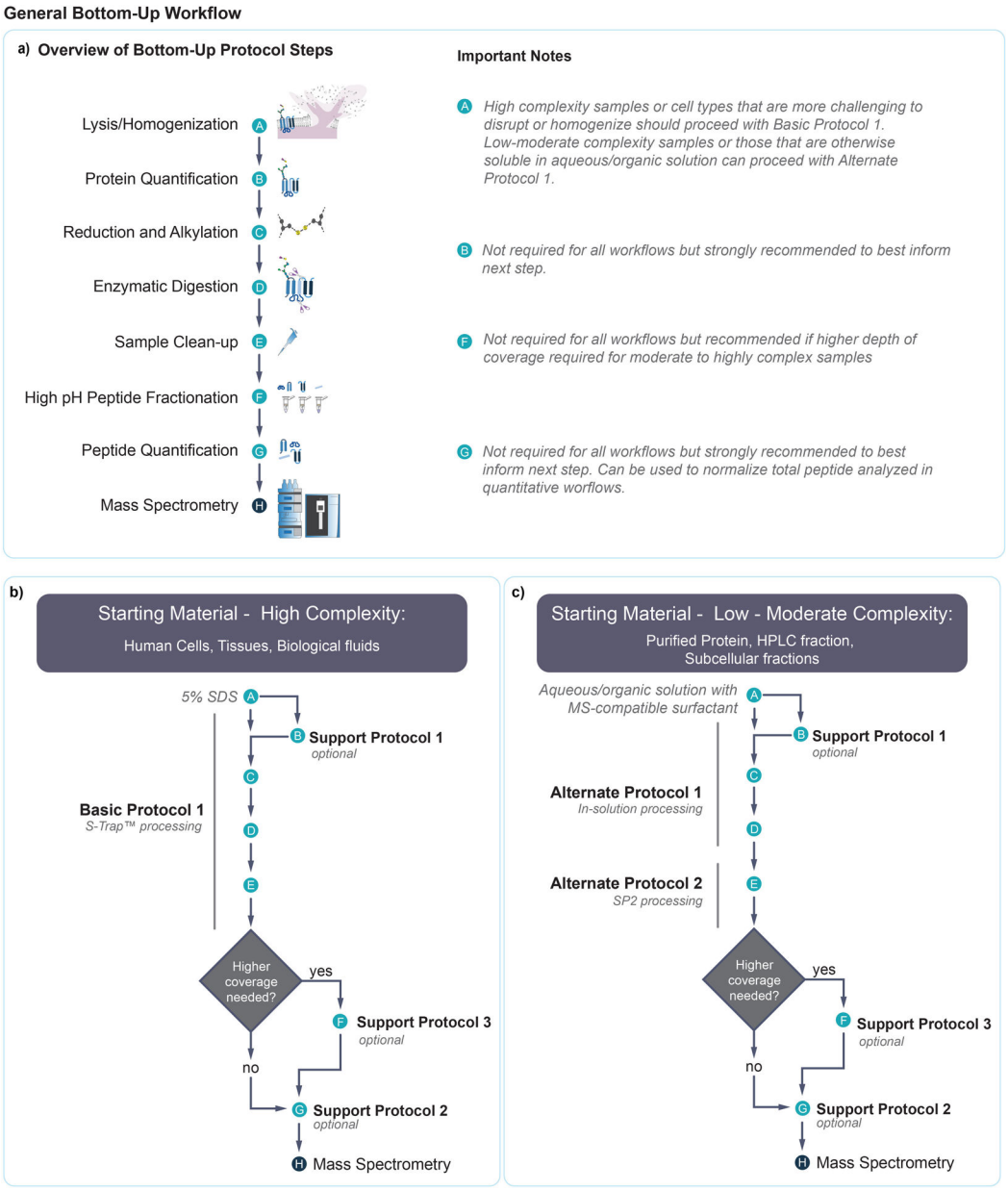

Figure 1. General Bottom-Up Workflow

a) Overview of a general bottom-up proteomics preparation workflow. Relevant notes for various steps are shown on the right. B) Overview of the workflow for processing high complexity samples, resulting from a combination of Basic Protocol 1 with Support Protocols 1, 2, and 3. C) Overview of the workflow for processing low to moderate complexity samples, resulting from a combination of Alternate Protocols 1 and 2, together with Support Protocols 1, 2, and 3. For B and C, examples of sample types appropriate for each workflow are provided.

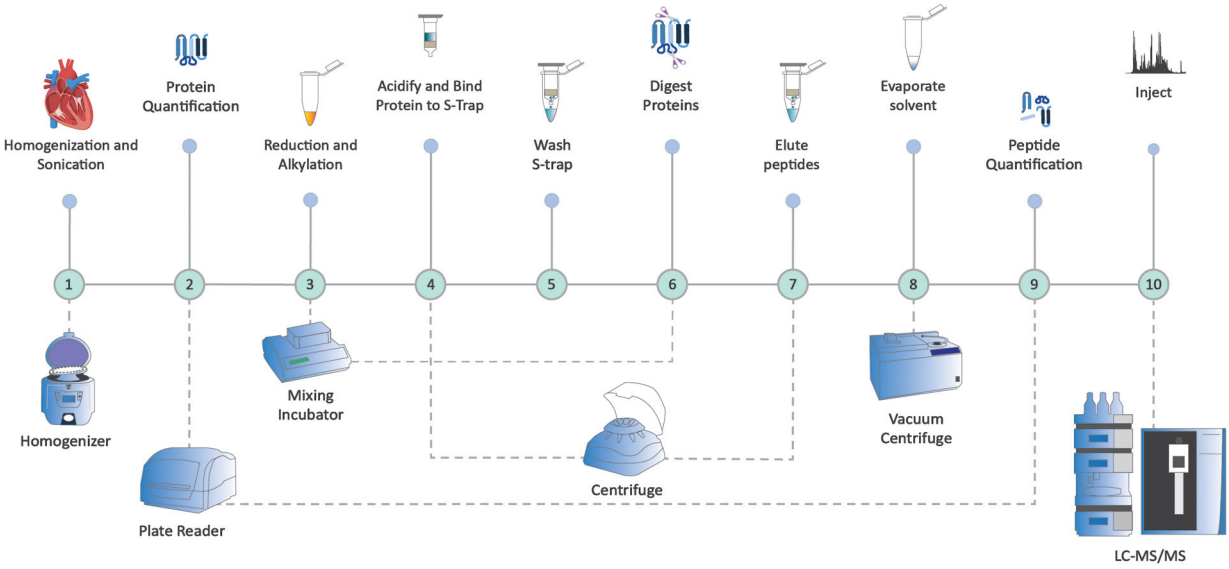

Figure 2. Overview of a bottom-up proteomics preparation workflow for high complexity samples using S-Trap™ processing, following Basic Protocol 1, and Support Protocols 1 and 2.

The sample is homogenized and sonicated prior to protein quantification. Disulfide bonds are reduced, and free thiols are alkylated. The sample is acidified, bound onto the S-trap™, and then washed free of contaminants. Digestion takes place on the spin column. Peptides are eluted, solvent evaporated, and peptides are quantified.

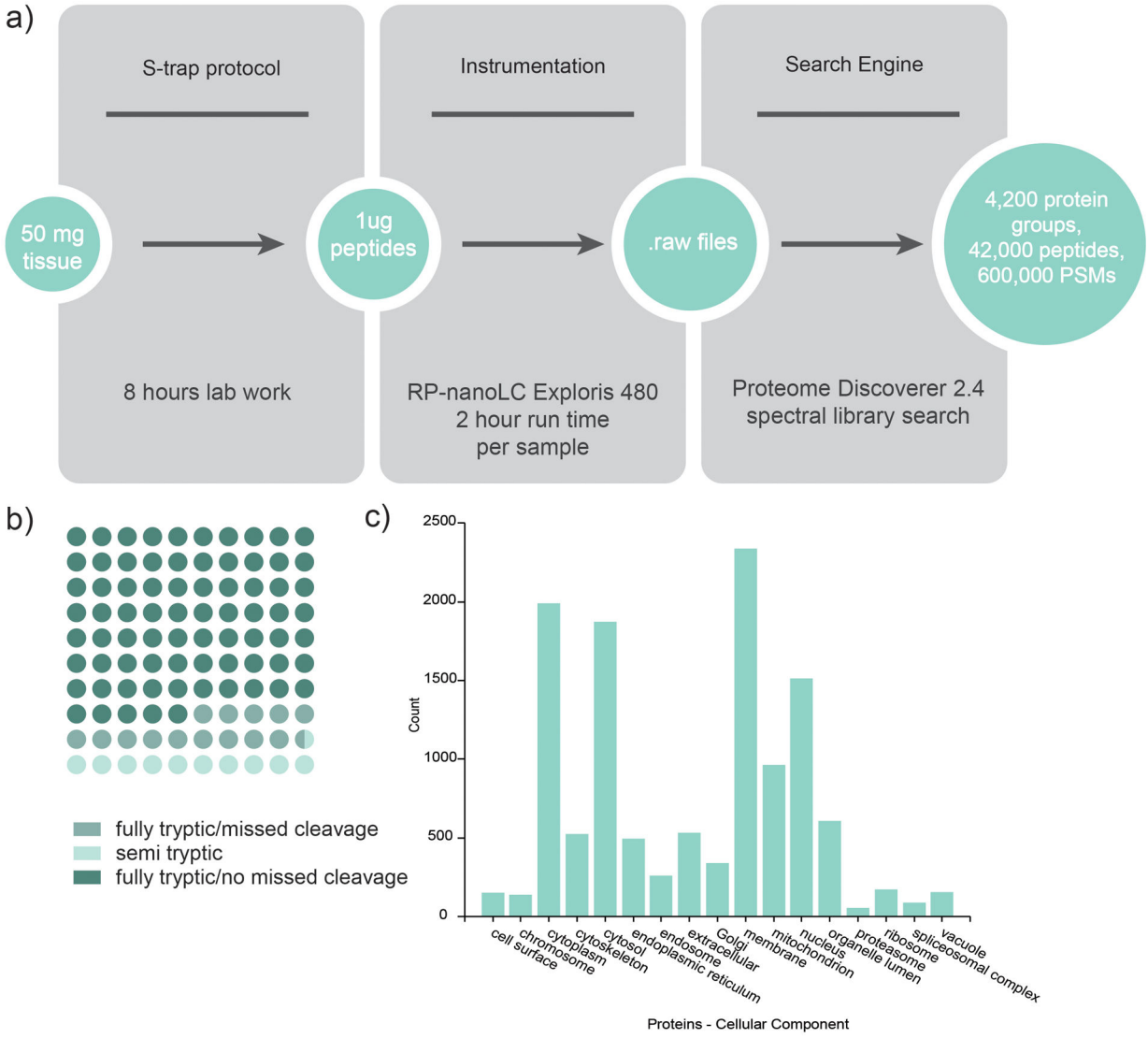

Figure 3. Sample data for Basic Protocol 1:

A) Protocol Workflow: S-trap™ processing of 50 mg of human heart tissue yields sufficient peptide material for 30–40 injections of 1 µg per injection. This workflow yields more than 4,000 proteins per injection. B) dot plot of percentage of peptides identified as fully tryptic with and without missed cleavages or semi-tryptic. C) Number of proteins annotated among various cellular components based on Gene Ontology as annotated by ProteomeDiscoverer 2.4.

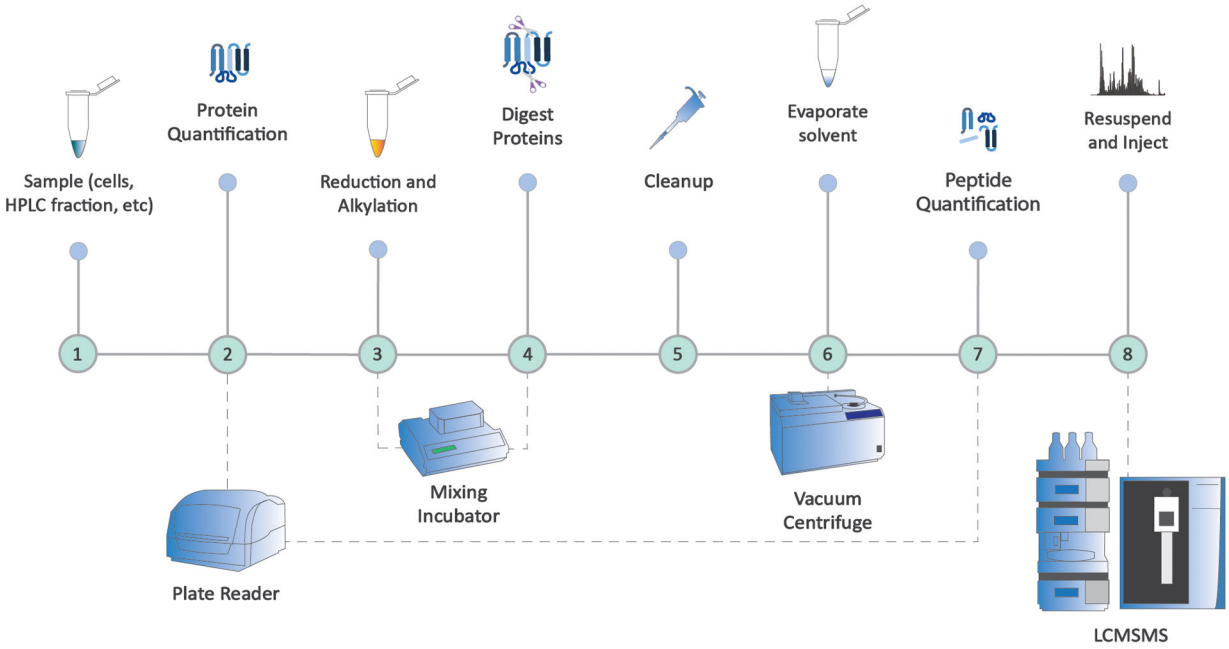

Figure 4. Overview of a bottom-up proteomics preparation workflow for low complexity samples, using Alternate Protocol 1 followed by Alternate Protocol 2, also including Support Protocols 1 and 2.

Protein quantity is first determined. Then, disulfide bonds are reduced and free thiols are alkylated. Proteins are digested enzymatically, contaminants are removed, and solvents are evaporated before peptide quantification.

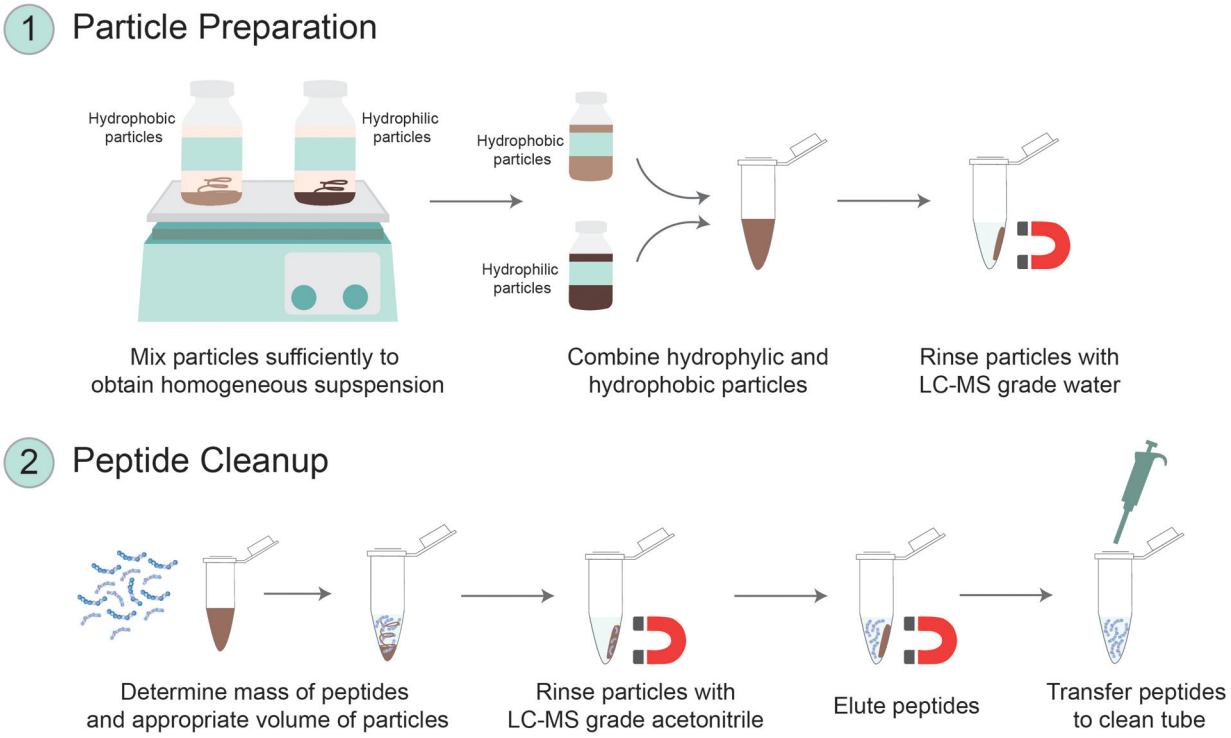

Figure 5. Overview of the SP2 protocol for detergent and salt removal from peptide samples using Alternate Protocol 2.

During particle preparation (1), hydrophobic and hydrophilic Sera-Mag SpeedBeads carboxylate-modified particles are combined and washed for subsequent use during peptide cleanup. (2) Particles are then combined with an appropriate mass of peptides followed by elution of cleaned peptides.

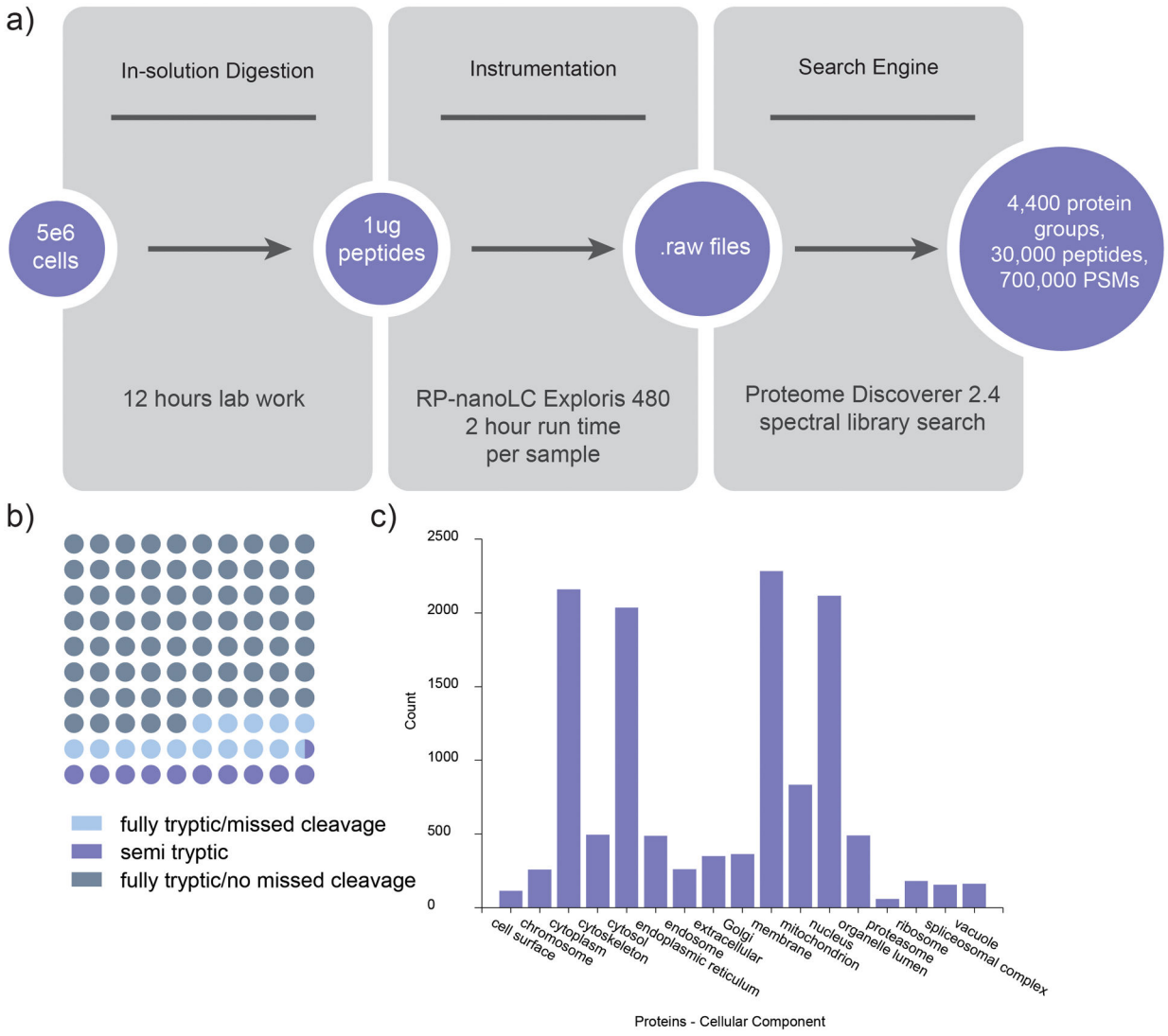

Figure 6. Sample data for Alternate Protocol 1 followed by Alternate Protocol 2:
a) Protocol Workflow: In-solution digestion of 5e6 RMPI 1788 cells followed by SP2 cleanup of the sample yields sufficient peptide material for 50 injections of 1 µg per injection. This workflow yields more than 4,000 proteins per injection. b) dot plot of percentage of peptides identified as fully tryptic with and without missed cleavages or semi-tryptic, c) Number of proteins annotated among various cellular components based on Gene Ontology as annotated by ProteomeDiscoverer 2.4.

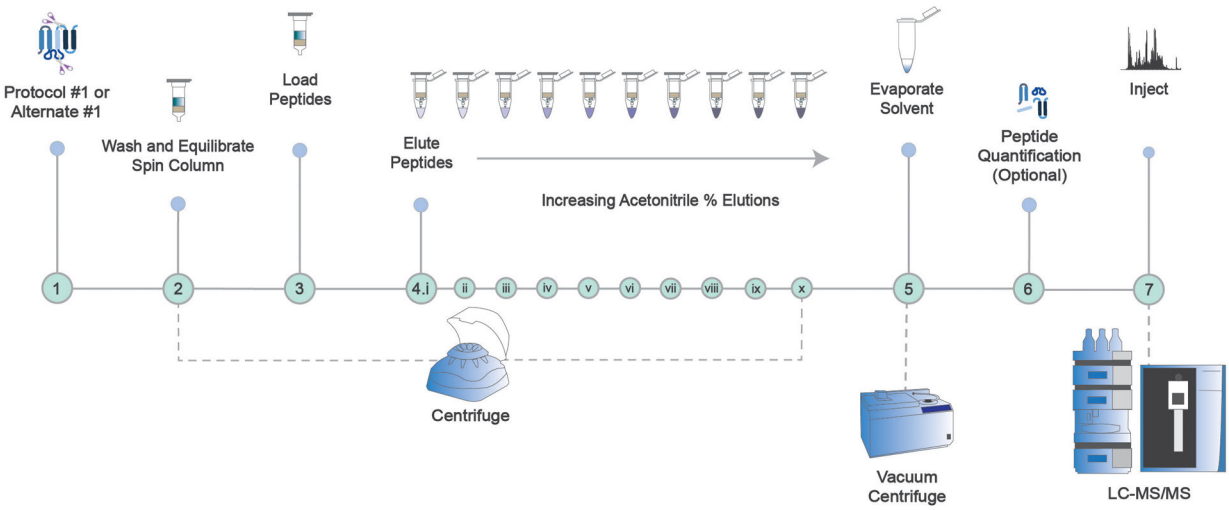

Figure 7. Overview of the protocol for high pH fractionation of peptide samples to obtain deeper coverage of the proteome for highly complex samples.

After performing Alternate Protocol 1 or 2, the resulting sample is then loaded onto a washed and equilibrated spin column and peptides are eluted with increasing amounts of acetonitrile, at a high pH. Solvent is evaporated and peptides are then quantified before MS analysis.

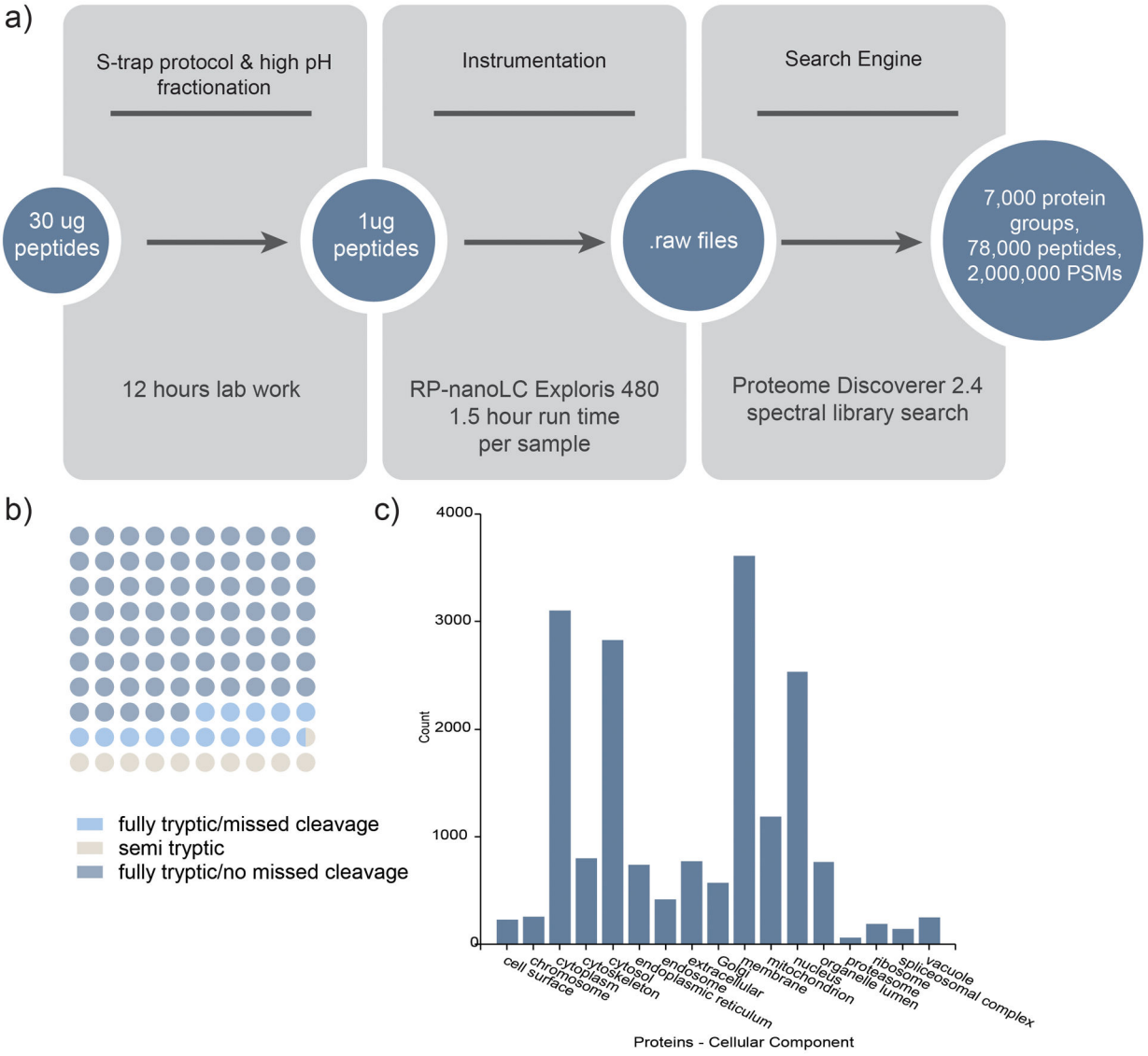

Figure 8. Sample data for Basic Protocol 1 followed by Support Protocol 3:

a) S-trap™ processing of human heart tissue followed by high pH fractionation to increase proteome coverage yielded 7,000 proteins. The protocol workflow and results by step are shown. B) dot plot of percentage of peptides identified as fully tryptic with and without missed cleavages or semi-tryptic. C) Number of proteins annotated among various cellular components based on Gene Ontology.

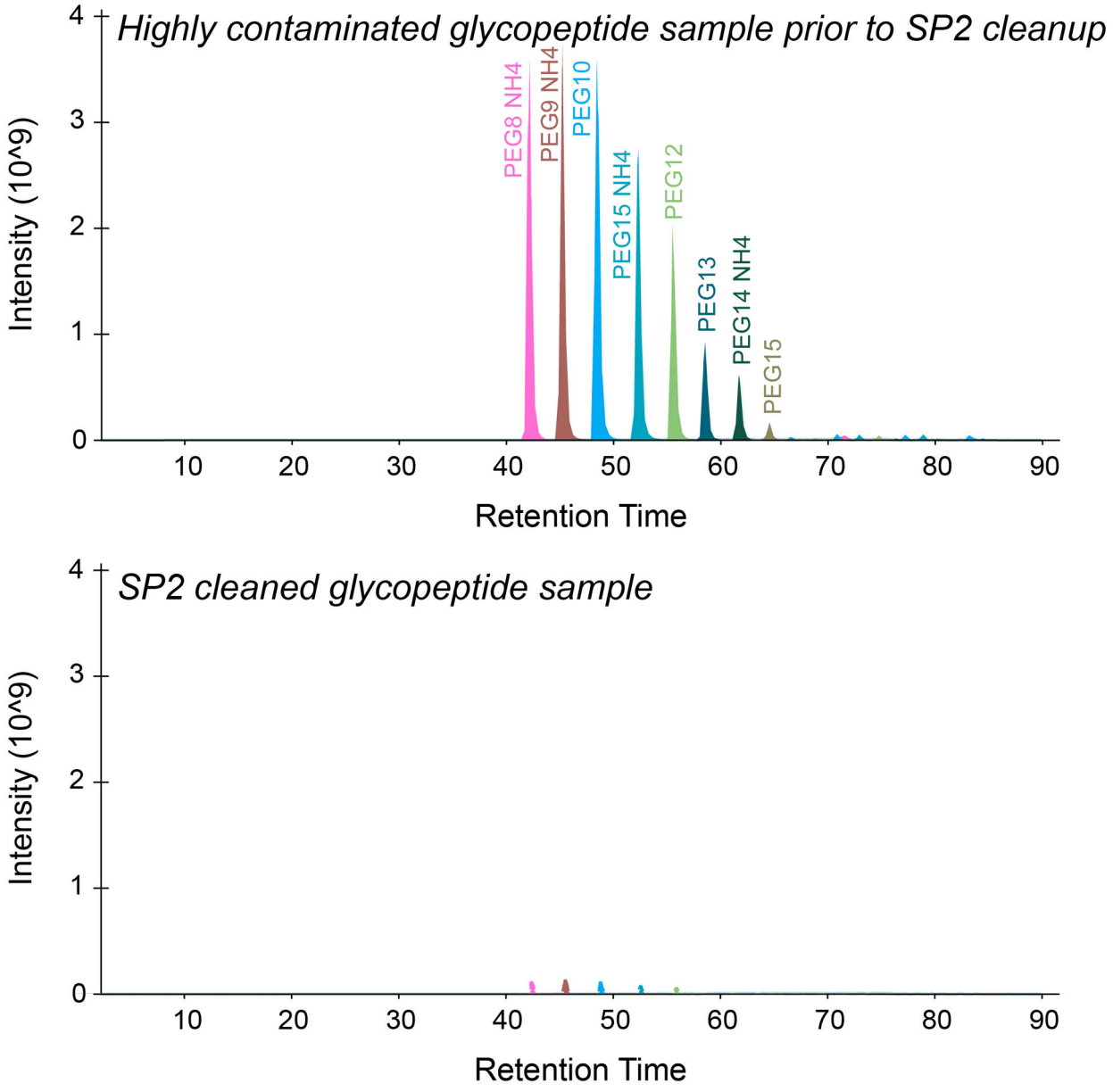

Figure 9. Base peak chromatogram for PEG contaminants prior to and after SP2 cleanup of a peptide sample.

Prior to SP2 cleanup (Alternate Protocol 2) the sample showed high levels of PEG contaminants (top panel), which are greatly reduced after SP2 cleanup (bottom panel).

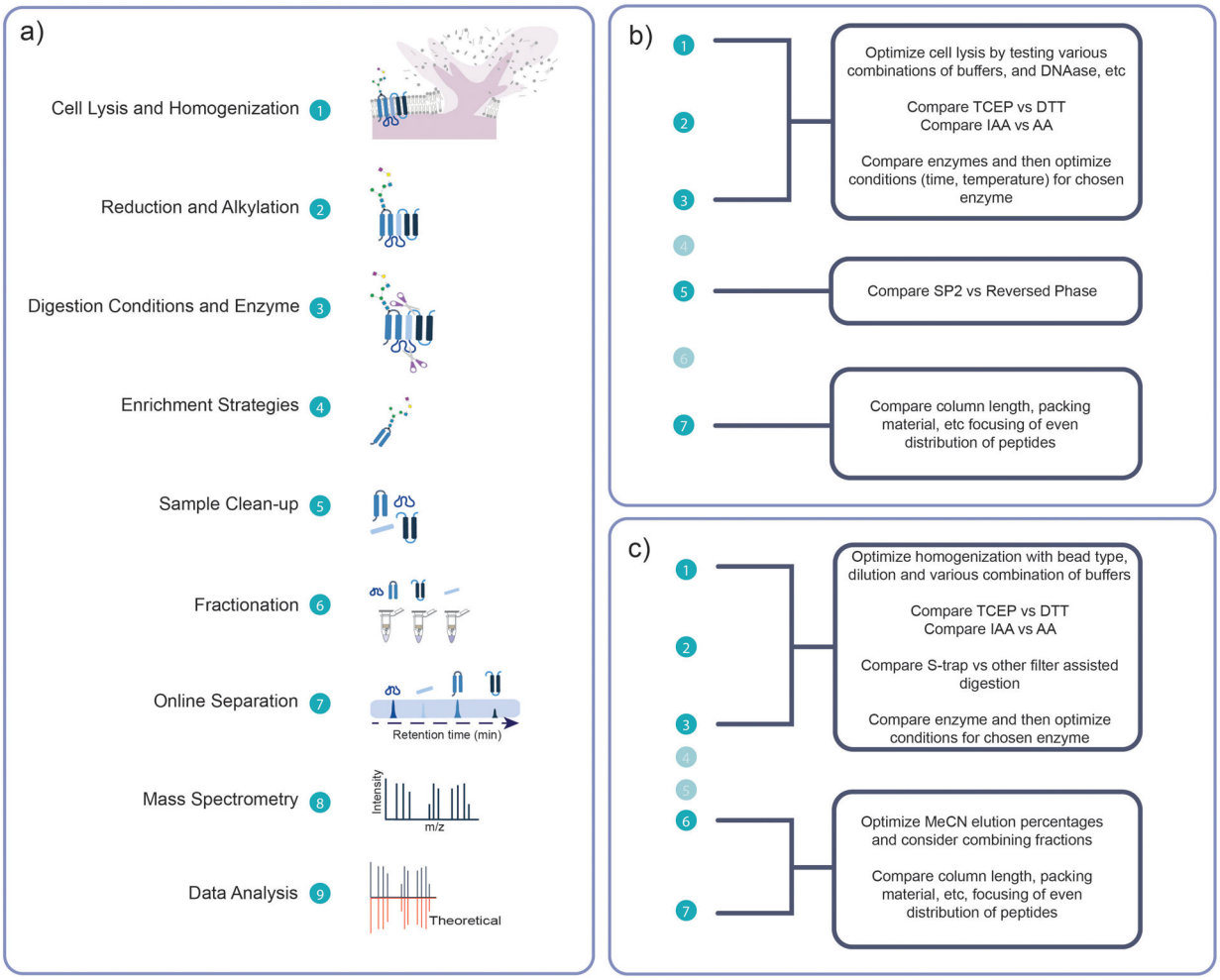

Figure 10. Bottom-up workflow method optimization;

a) Various steps within a bottom-up proteomics workflow that can be optimized according to sample type and experimental goals. b) Example of sample preparation method optimization using Alternate Protocol 1 for in-solution processing of cells. C) Example of sample preparation method optimization using Basic Protocol 1 for S-trap™ processing of tissue.

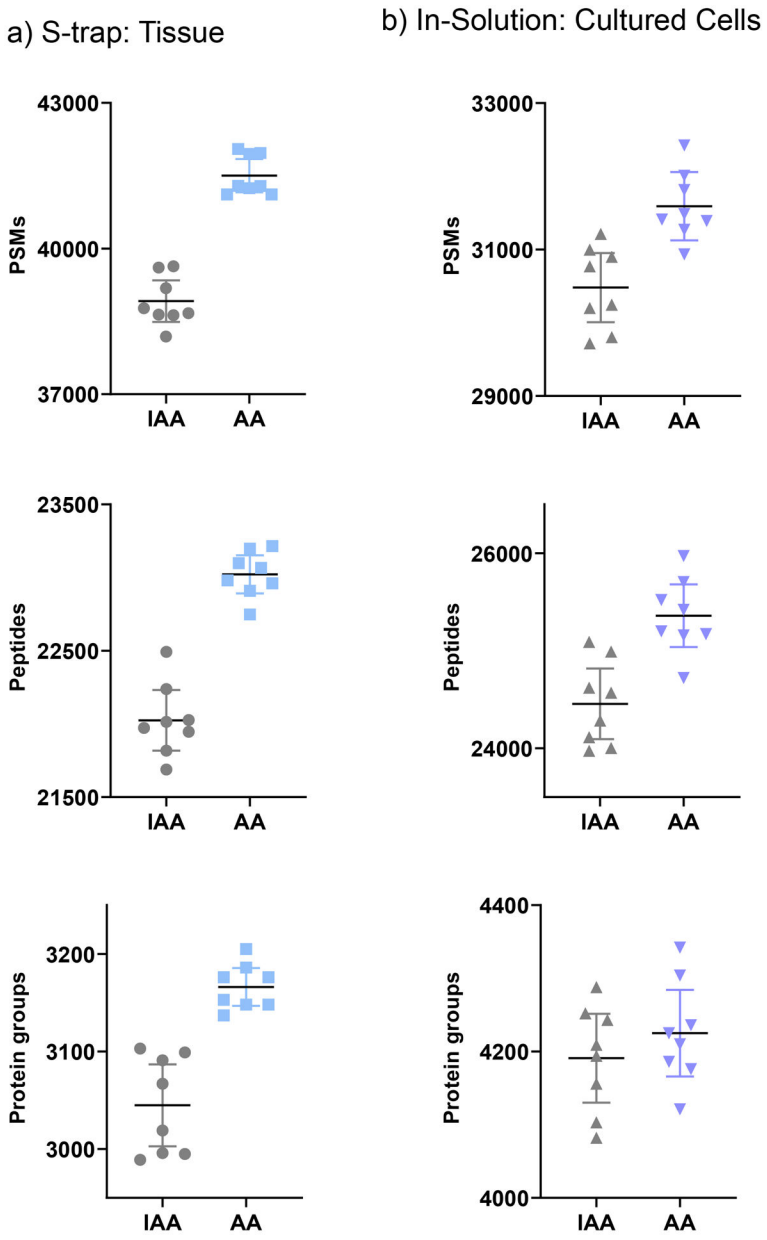

Figure 11. Number of protein groups, peptides, and peptide spectrum matches (PSMs) when using iodoacetamide (IAA) or acrylamide (AA) as alkylating reagents.

a) Results of IAA and then AA performed with Basic Protocol 1 S-trap™ processing of human heart tissue. B) Results of IAA and AA performed with Alternate Protocol 1 in solution processing of RPMI 1788 cell lysis. Shown are data for 8 technical replicates. Line represents the mean, and error bars, 95% confidence interval.

Table 1.

Examples of protein quantity values for various cell and tissue types obtained using the Pierce™ 660 nm assay.

Sample	Typical Protein Quantity, µg
1 million human B cells (RPMI 1788)	100
1 million human embryonic kidney cells (HEK)	200
1 million human pluripotent stem cells	300–400
50 mg wet weight human heart tissue, ventricle	1000

Table 2.

Summary of common problems encountered and their solutions.

Problem	Solution
Basic Protocol 1	
Sample clogged on S-Trap™	Centrifuge the sample thoroughly before pipetting and be sure to shear DNA with sonication or with the addition of $MgCl_2$ and Benzonase.
Contaminants in MS data such as polyethylene glycol, lipids, or high backpressure during sample loading	Perform additional wash steps on S-trap™ at step 14. For removal of lipids, add 3 washes of 50:50 methanol:chloroform before standard S-trap™ buffer washes
Percentage of peptides identified with at least 1 missed cleavage is greater than 15%	Increase the concentration of trypsin if digesting a large amount of protein.
Alternate Protocol 1	
Sample contaminated with detergents or polymers as evidenced by poor LCMS data	Use detergent free glassware; be sure to perform SP2 cleanup, as C_{18} cleanup protocols do not remove PEG
Large number of missed cleavages in database search results	Check that the digestion solution pH is ~8 after adding trypsin to the protein and adjust with small amounts of concentrated acid or base as necessary.
Alternate Protocol 2	
Low peptide recovery	During particle preparation, let particles warm to room temperature under constant shaking on an orbital shaker for at least 30 minutes. Sufficiently suspend particles by shaking and vortexing to yield a homogenous suspension. Particle preparation should be made fresh every 6 month. Ensure that math was performed correctly during peptide quantification assay. Alternatively, repeat peptide quantification assay. To maximize recovery, use 0.2–0.4 µL of 50 µg/µL particle suspension per µg of peptide to be cleaned, which corresponds to a particle to peptide ratio of 10:1 to 20:1.
PEG or other contaminants in MS data	Use detergent free glassware. Make fresh elution solution.
Support Protocols 1, 2	
No protein in sample or low protein concentration after lysis	Use ionic detergent compatibility reagent included with the Pierce kit.
Variable numbers in duplicate values	Be sure to allow the reagents to come to room temperature. Consider checking pipette technique.
No protein in sample or low protein concentration after tissue lysis	Be sure to use ionic detergent compatibility reagent included in the Pierce kit if there are detergents in the sample.
Too much protein for standard range	Dilute sample further with lysis buffer before attempting protein quantification again.
Support Protocol 3	
Variable peptide amounts in fractions	Consider changing acetonitrile percentages to optimize spread of peptides.
No peptides in any fractions	Check the saved “flow through” and wash tubes for un-retained peptides.