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Proteome Analysis Using Gel-LC-MS/MS

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Abstract

This manuscript describes processing of protein samples using 1D SDS gels prior to protease digestion for proteomics workflows that subsequently utilize reversed-phase nanocapillary ultra-high pressure liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS). The resulting LC-MS/MS data are used to identify peptides and thereby infer proteins present in samples ranging from simple mixtures to very complex proteomes. Bottom-up proteome studies usually involve quantitative comparisons across several or many samples. For either situation, 1D SDS gels represent a simple, widely available technique that can be used to either fractionate complex proteomes or rapidly clean up low microgram samples with minimal losses. After gel separation and staining/destaining, appropriate gel slices are excised, in-gel reduction, alkylation, and protease digestion are performed, and digests are processed for LC-MS/MS analysis. Protocols are described for either sample fractionation with high throughput processing of many samples or simple cleanup without fractionation. An optional strategy is to conduct in-solution reduction and alkylation prior to running gels, which is advantageous when a large number of samples will be separated into large numbers of fractions. Optimization of trypsin digestion parameters and comparison to in-solution protease digestion are also described.

Keywords

proteomics; Gel-LC-MS/MS; SDS gels; in-gel digestion; mass spectrometry; LC-MS/MS; proteome fractionation

INTRODUCTION

1D SDS gels (Gallagher, 2012) are a powerful separation technique that can be combined with bottom-up proteomics analysis (Wither, Hansen, & Reisz, 2016). This approach, termed Gel-LC-MS/MS or GeLC-MS/MS, is compatible with commonly used quantitative protein profile comparison approaches, particularly stable isotope labeling with amino acids in cell

culture (SILAC) (Deng, Erdjument-Bromage, & Neubert, 2019; Ong et al., 2002) and label-free quantitation (LFQ) (Cox et al., 2014). A general overview of the analysis strategy and relationships of the included protocols to each other is depicted in Figure 1. As described in the Basic Protocol, 1D SDS gels can be used to separate complex samples based on molecular weight (MW). After fixing and staining the gel, typically with colloidal Coomassie, the gel lane is separated into equal gel slices and each slice is subjected to in-gel protease digestion followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Monteoliva & Albar, 2004). For complex samples with a large dynamic range of protein abundances, such as plasma ($>10^{10}$) (Anderson & Anderson, 2002), immunodepleting abundant proteins, running short-distance gels, and fractionating the separation region prior to in-gel digestion increases depth of analysis (Beer, Ky, Barnhart, & Speicher, 2017). Even for samples with smaller dynamic ranges, such as human cell lines or tissue, 1D gel fractionation, in-gel digestion and subsequent analysis of each fraction using moderate length reversed-phase gradients, e.g. 70–90 min, will usually increase the number of confidently identified peptides and proteins. For example, previous studies analyzing cancer cell line lysates have identified >8000 proteins using gel-based fractionation (Gholami et al., 2013), which represents about 80% of the expressed genes.

A second application of 1D SDS gels is to clean up samples for bottom-up proteomics when fractionation is not required (Alternative Protocol). This approach is particularly useful if the sample contains SDS or other MS-incompatible detergents, buffers or salts. In addition, including SDS in the sample extraction buffer will usually result in more effective solubilization of a broad range of proteins when lysing cells or tumors, including membrane proteins, that may be poorly solubilized by other methods. Use of 1D gels is also particularly advantageous when working with small amounts of total protein, e.g. less than 10 micrograms, because these samples can be partially or completely lost during in-solution processing, unless reagents such as SDS that prevents adsorption are present. Sample cleanup using 1D gels is typically accomplished by electrophoresing the sample into the gel for only about 0.5 cm. A short gel separation is desirable for this application as this minimizes the total gel volume in the in-gel digestion. A large gel volume to total protein ratio tends to decrease both digestion efficiency and peptide recovery after digestion. This one fraction or “one-shot” approach, when coupled with a longer (up to 4 hr) LC gradient and a modern, high speed, high resolution, high mass accuracy mass spectrometer (e.g. Thermo Q Exactive series mass spectrometers or their equivalent) can yield excellent depth of analysis in a single fraction. Typically, on the order of 4,000–5,000 proteins per cancer cell lysate or 700 proteins in major protein-depleted plasma can be identified using a single, 4 hr gradient (Figure 2A and 3A).

These protocols are commonly used in proteomics analyses and combine reproducible protein separation methods with well-established sensitivity of in-gel digestions followed by LC-MS/MS for protein identifications (Beer, Tang, Barnhart, & Speicher, 2011). Our laboratory has used Gel-LC-MS/MS to analyze the proteomes of a variety of sample types, including cellular extracts (Goldman et al., 2016), plasma (Beer et al., 2013), conditioned media from cell culture and tumor samples (Kraya et al., 2015), and erythrocyte membrane cytoskeletal proteins (Rivera-Santiago, Harper, Sriswasdi, Hembach, & Speicher, 2017). These protocols can be used to analyze small numbers of samples where in-gel digests are

typically performed using 0.5 ml microfuge tubes. Larger numbers of samples, particularly when fractionation of multiple samples will be pursued can be efficiently processed using a simple 96-well pierced plate format and an 8-channel pipette. In some cases, it may be desirable to reduce and alkylate samples prior to 1D SDS gel electrophoresis as this eliminates the need to perform these steps on each in-gel digest sample (Support Protocol).

STRATEGIC PLANNING

Four major factors to consider when planning a Gel-LC-MS/MS experiment include: 1) how much total protein per sample needs to be digested, 2) is proteome fractionation needed and, if so, how many fractions per sample are needed to achieve the desired depth of analysis, 3) how many digests are being performed and should the 96-well pierced plate approach be used to increase throughput, and 4) what is the optimal gradient length that should be used in the LC-MS/MS analysis.

The optimal (maximum) amount of tryptic digestion from a cell lysate that should be injected on a 75 μm internal diameter (i.d.) column is about 1.0 μg for most LC-MS systems. This means that a 5 μg load on a gel with 0.5 cm separation (no fractionation) will provide adequate digested sample even if a re-analysis is needed due to instrument performance problems. If fractionation is required, about 50 μg of a complex sample such as a cancer cell lysate can be separated on 1.0 mm mini-gels with 10 wells without over-loading the gel and causing excessive band smearing. Therefore, due to the high sensitivity of most current LC-MS/MS systems that use nanocapillary UPLC columns (e.g. 75 μm i.d.), one or two lanes per sample will provide an adequate amount of protein for most protein identification/quantitation experiments. For example, if one 50 μg lane is sliced into 10 fractions, each fraction will contain an average of 5 μg digested peptides, which is sufficient for multiple LC-MS/MS runs per fraction. The most common situation where 1D gels do not have adequate capacity is if digested samples will be used to enrich for specific posttranslationally modified peptides, such as phosphopeptides, acetylated peptides, or ubiquitin-modified peptides. Due to the low stoichiometry of most posttranslational modifications (PTMs), most PTM enrichment protocols start with 200 μg or more of a digest. Therefore, in-solution digests are typically preferred for PTM enrichment.

Whether sample fractionation is needed and, if so, the appropriate extent of sample fractionation required is primarily a tradeoff between instrument time and depth of analysis. Fractionation is particularly advantageous for increasing protein identifications for complex samples, such as mammalian cell or tissue extracts, and biological fluids, such as serum or plasma. The number of fractions needed per sample will determine how far samples should be allowed to electrophorese. As noted above, the gel volume to protein ratio per in-gel digestion reaction should be kept relatively low to ensure good digestion efficiency and recovery of peptides from gel slices. Running samples for a short distance, typically 2.0 to 4.0 cm, is optimal depending upon the selected number of fractions. The Basic Protocol outlines a method where gel lanes are cut into 1.0 mm slices and the trypsin digestion conditions are optimized for digestion of 1 to 3 slices per reaction (Figure 4A). Therefore, if samples are electrophoresed for 2.0 cm, three common alternative options for setting up the digests are: 1) a single lane per sample can be digested in 20 separate reactions resulting in

20 LC-MS/MS runs per sample; 2) sequential slices in a single lane are combined for 2-slice digests, e.g. slice 1, 2 = digest 1, slice 3, 4 = digest 2, ... slice 19, 20 = digest 10; or 3) corresponding slices from two or three replicate lanes of a sample can be combined in the same digestion reaction to produce 20 fractions with two or three times as much peptide amount per digest, respectively. For most gel systems and sample types, electrophoresing for at least 2 cm will give better size fractionation than a shorter separation. A variation on the above strategies for pooling gel slices prior to in-gel digestion is to pool appropriate fractions post-digestion. Furthermore, both pooling strategies can be combined. For example, run replicate aliquots of a sample in three adjacent lanes, combine the three slice 1 samples in digest 1, slice 2 samples in digest 2, ... slice 20 samples in digest 20. After digestion, every two adjacent digests can be combined and lyophilized to produce a 10-fraction proteome for applications where larger amounts of digests are needed for each fraction. Refer to the Basic Protocol if fractionation is needed and the Alternative Protocol if samples are being processed without fractionation (0.5 cm gel, Figure 4B).

The total number of digestions to be performed simultaneously (total number of samples x total number of digestions per sample) will dictate certain protocol steps. If the total number of digests is small, e.g. less than 20 discrete digestions, performing the digests in 0.5 ml microcentrifuge tubes works well. However, for much larger numbers of digests, throughput can be substantially increased, and the likelihood of errors decreased, by using 96-well pierced plates and an 8-channel pipette for reagent additions. Using this setup, a single experienced operator can readily perform up to 192 (two plates) and possibly 384 (four plates) digests at a time. An example of such an experiment would be to process 18 samples with 10 digests per sample, or 180 total digests. In situations such as this one where 10 or more in-gel digests will be performed per sample, it may be more efficient to reduce and alkylate each sample prior to 1D SDS gel electrophoresis (Support Protocol) as this will save multiple steps in the in-gel digestion method.

Finally, the length of the LC-MS/MS run that is most time efficient will depend upon the number of fractions per sample to be analyzed. We have found that for our workflow with Q Exactive mass spectrometers and a 75 μ m C18 column with 1.7 μ m particles the optimal length LC gradient for a single shot (single fraction) proteome analysis is on the order of 4 hr (Figures 2A and 3A). In contrast, when proteomes are divided into five or more fractions a gradient on the order of 70 to 90 min is the best compromise between total instrument time per sample and overall depth of analysis.

BASIC PROTOCOL

LARGE SCALE IN-GEL FRACTIONATION AND TRYPSIN DIGESTION

In this protocol, samples are separated by 1D SDS gel electrophoresis, gels are stained with colloidal Coomassie, and gel lanes are uniformly cut into 1.0 mm slices then reduced, alkylated, and digested in-gel by trypsin. Samples are processed in 96-well plates that have been pierced with small laser-cut holes at the bottom of the wells. Aqueous samples are retained due to surface tension, but liquid can be rapidly and efficiently removed by a 1 min centrifugation at room temperature in a bench top centrifuge ($\sim 300 \times g$). Reagents are added using an eight-channel pipette. To minimize airborne keratin contamination, gel cutting,

transfer of gel slices to the 96-well plate, and addition of reagents should be performed in a laminar flow PCR hood equipped with a light box. The 96-well plates are covered by cleaned polystyrene plate covers whenever they are removed from the PCR hood to further limit airborne contamination. Also, prior to use, all plasticware including HPLC autosampler tubes should be prewashed twice with 0.1% (v/v) trifluoroacetic acid, 50% (v/v) methanol in water, rinsed twice with methanol, and air dried in the PCR hood to reduce contaminating polymer peaks in MS spectra. Similarly, forceps, razor blades and other tools that contact gels or digests should be cleaned thoroughly using methanol followed by Milli-Q water. If the number of samples to be processed is modest, e.g. 20 or less, this protocol can be performed using 0.5 ml microcentrifuge tubes rather than 96-well plates. In this case, liquid needs to be removed from gel pieces using gel-loading pipette tips. A general overview of the protocol is depicted in Figure 1.

Materials—Sample(s) containing protein(s) of interest

Plasticware Wash Buffer: 0.1% (v/v) trifluoroacetic acid, 50% (v/v) methanol (see recipe in Reagents and Solutions)

Optima™ Methanol, 0.2 µm filtered (Thermo Fisher Scientific)

SDS Protein Solubilizing Buffer (5X): 1 M sucrose, 15% (w/v) SDS, 312.5 mM Tris-Cl, 10 mM Na₂EDTA, 5% (v/v) 2-mercaptoethanol, 5% (v/v) saturated bromophenol blue (see recipe in Reagents and Solutions), pH 6.9

MES Running Buffer (1X): 50 mM MES, 50 mM Tris Base, 0.1% (v/v) SDS, 1 mM Na₂EDTA, pH 7.3 (see recipe in Reagents and Solutions)

NuPAGE® 10% Bis-Tris gels, 1 mm, 10-well or 15-well (Thermo Fisher Scientific)

3/10 cc insulin syringe (Becton Dickinson)

India ink

Gel Fixing Solution: 50% (v/v) methanol, 10% (v/v) acetic acid (see recipe in Reagents and Solutions)

Novex® Colloidal Blue Staining Kit (Thermo Fisher Scientific) with Stainer A and Stainer B Staining Solution (see recipe in Reagents and Solutions)

0.4 M ammonium bicarbonate, pH 8.0 (see recipe in Reagents and Solutions)

Destaining Solution: 0.2 M ammonium bicarbonate, 50% (v/v) acetonitrile (see recipe in Reagents and Solutions)

Reducing Solution: 20 mM tris(2-carboxyethyl) phosphine hydrochloride, 25 mM ammonium bicarbonate (see recipe in Reagents and Solutions)

Alkylating Solution: 40 mM iodoacetamide, 25 mM ammonium bicarbonate (see recipe in Reagents and Solutions)

Wash Buffer 1: 25 mM ammonium bicarbonate (see recipe in Reagents and Solutions)

Wash Buffer 2: 25 mM ammonium bicarbonate, 50% (v/v) acetonitrile (see recipe in Reagents and Solutions)

Trypsin Working Solution: 4 ng/μl trypsin, 40 mM ammonium bicarbonate (see recipe in Reagents and Solutions)

Digest Extraction Buffer: 4.5% (v/v) neat formic acid, 40 mM ammonium bicarbonate (see recipe in Reagents and Solutions)

Acetonitrile Extraction Buffer: 80% (v/v) acetonitrile (see recipe in Reagents and Solutions)

Digest Resuspension Buffer: 0.1% (v/v) neat formic acid, 4% (v/v) acetonitrile (see recipe in Reagents and Solutions)

Heat block set to 90 °C

XCell SureLock™ Mini-Cell Electrophoresis System (Thermo Fisher Scientific), Mini Gel Tank (Thermo Fisher Scientific) or comparable system

Staining trays

Platform shaker

PCR laminar flow hood equipped with HEPA filter

Lightbox (sized to fit inside PCR Hood)

Glass plate to cover light box (~20 cm × 22 cm)

Polystyrene plate cover

Glass plate cut to dimensions of 96-well plate (8 cm × 12 cm)

Gel cutting device that can cut gel lanes into precise 1 mm slices (e.g. The Gel Company or custom razor blade array)

96-well pierced plate with V-shaped well bottoms and with laser-cut holes; e.g. LVL Digestion Plate (LVL Technologies) or Perforated Plates (GlySci)

96-well collecting plates with V-shaped well bottoms (e.g. Thermo Fisher Scientific)

Stainless-steel razor blades

Forceps

Ziploc® bags (or generic alternative)

SpeedVac™ centrifuge with 96-well plate rotor (Thermo Fisher Scientific)

Eight-channel pipette

“Dry” temperature-controlled incubator/shaker unit set to 37 °C (e.g., Taitec M-36 Microincubator)

Autosampler tubes (e.g. Thermo Fisher Scientific)

1D SDS Gel Electrophoresis

1. Use a permanent marker to mark the surface of a 10% Bis-Tris, 1 mm, 10-well NuPAGE[®] gel cassette at predetermined sample separation distance (e.g. 2 cm) by measuring from bottom of wells.

Separation distance is dependent on the number of fractions that are required for LC-MS/MS analysis with an appropriate depth of analysis. Gel lanes are sliced uniformly every 1.0 mm (e.g. a 2.0 cm sample separation yields 20 × 1.0 mm slices). Depending on the desired number of fractions and total amount of digested peptides per fraction, up to 3 slices can be combined per digest using either the same slice from replicate lanes or sequential slices within a lane (see Strategic Planning).

2. Add SDS Protein Solubilizing Buffer (5X) to protein samples such that final concentration is 1X.

Well volumes of 1.0 mm, 10-well NuPAGE[®] gels used in this protocol are about 25 µl. Therefore, the maximum sample load is 20 µl plus 5 µl 5X Solubilizing Buffer. Alternatively, 1.0 mm, 15-well gels can be used but maximum sample load is about 12 µl plus 3 µl 5X Solubilizing Buffer.

3. Heat samples for 2 min at 90 °C.
4. Assemble XCell SureLock[™] Mini-Cell unit, and add 1X MES Running Buffer to both chambers.
5. Load molecular weight standard (e.g. Benchmark[™] Protein Ladder, Thermo Fisher Scientific) and samples into appropriate lanes.
6. Run gel apparatus at 200 V using constant voltage.
7. Stop run when the dye front has migrated the predetermined separation distance.
8. Disassemble unit and remove gel from cassette.
9. Using a syringe dipped in India Ink, mark the outer edges of the gel to indicate the precise migration of the dye front. Also, cut diagonally across the bottom left corner of the gel for proper orientation.
10. Transfer gel to staining tray containing 100 ml Fixing Solution.
11. Incubate gel for 10 min with gentle shaking on a platform shaker.
12. Decant Fixing Solution and add 95 ml Staining Solution to tray.

13. Incubate gel in Staining Solution for 10 min with shaking, then add 5 ml Stainer B to tray and incubate 3 hr or overnight with shaking.
14. Decant Staining Solution, add 200 ml Milli-Q® water, and incubate overnight with shaking to destain gel.

The destain process can be expedited by changing water periodically.
15. Carefully transfer gel to a Ziploc® bag, scan for recordkeeping purposes, and store at 4 °C until ready to perform in-gel digest.

In-gel Reduction, Alkylation, and Digestion

16. Turn on PCR hood at least 15 min before cutting gels to achieve optimal air flow.
17. Start-up SpeedVac™ system.
18. Place pre-cleaned, 96-well pierced plate on top of 96-well collecting plate.
19. Add 100 µl/well Milli-Q® water to the number of wells of the pierced plate needed for in-gel digests.

If running 0.5 cm gels for one-shot proteomes, go to Alternative Protocol, step 8. Otherwise, continue to next step.
20. Excise the sides of gel lane(s) of interest using a sharp, cleaned razor blade, and cut each lane horizontally into uniform, 1.0 mm thick gel slices (see Figure 4A) using a razor blade array, or commercial gel-cutting device.

Cut gel on top of a lightbox protected by a glass plate. Rinse working surface and cutting tools with methanol followed by Milli-Q® water prior to cutting. Do not allow methanol to contact gel as this will result in gel dehydration. It is recommended to trim outer vertical lane edges with a razor blade before cutting gel into uniform horizontal slices to avoid edge effects; the edges of lanes often show vertical streaking indicative of poor protein separation at the lane edges (see Figure 4A).

For horizontal slices, we use a razor blade array constructed of stainless-steel razor blades separated by 1-mm Teflon spacers to slice gel lanes. A typical razor blade array produced in a local university machine shop is shown in Figure 5. This custom array is preferred to disposable commercial gel cutting grids because the blades are sharper than cutting grids, which partially mash the gel. Such small pieces of mashed polyacrylamide can readily cross-contaminate other fractions. Also, the 4 cm width of the razor blade array allows simultaneous slicing of multiple adjacent lanes, which increases reproducibility of gel slicing across lanes.

21. Using forceps, transfer gel slices to individual wells of a 96-well pierced plate that has been stacked on top of a V-bottomed 96-well collecting plate.

If the same sample is run in multiple gel lanes, equivalent fractions from these lanes (e.g. fraction 1 from all lanes) may be combined into the same wells. A

maximum of 3 gel slices/well is recommended as reagent volumes have been optimized for 1 to 3 gel slices that are 1 mm X 1 mm X ~4 mm. Larger gel volumes will increase retention of reagents with less effective washout as well as increased retention of digested peptides.

22. Centrifuge plates for 1 min at $300 \times g$, RT, to remove water, and discard liquid.
23. Destain gel slices with 100 μ l/well Destaining Solution.
24. Incubate 30 min at 37 °C with shaking.
25. Centrifuge plates for 1 min at $300 \times g$, RT, to remove Destaining Solution, and discard liquid.

If gel slices retain substantial blue color, repeat destaining step until they appear light blue or white. If samples have been reduced and alkylated prior to running 1D SDS gels using the Support Protocol, skip to Step 33 of this protocol after destaining.

26. Dry gel slices in SpeedVac™ for approximately 20 min.
27. Reduce proteins retained in gel slices by adding 100 μ l/well Reducing Solution and incubating for 15 min at 37 °C with shaking.
28. Centrifuge plates for 1 min at $300 \times g$, RT, to remove Reducing Solution, and discard liquid.
29. Alkylate proteins by adding 100 μ l/well Alkylation Solution and incubating for 30 min at 37 °C in the dark.

Cover incubator with tinfoil during incubation.

30. Centrifuge plates for 1 min at $300 \times g$, RT, to remove Alkylation Solution, and empty collecting plate.
31. Add 100 μ l/well Wash Buffer 1, and incubate for 15 min at 37 °C. Centrifuge plates for 1 min at $300 \times g$, RT, and empty collecting plate. Repeat this step an additional time.
32. Add 100 μ l/well Wash Buffer 2, and incubate for 15 min at 37 °C. Centrifuge plates for 1 min at $300 \times g$, RT, and empty collecting plate.
33. Dry gel slices in SpeedVac™ for approximately 20 min.

This is an appropriate stopping point if protocol cannot be completed in the same day. Place pierced plate on top of collecting plate, cover with polystyrene plate cover, seal in Ziploc® bag, and store at 4 °C overnight.

34. Next day: turn on PCR hood at least 15 min before continuing protocol and start-up SpeedVac™ system.
35. Place 96-well pierced plate on top of a clean V-bottomed 96-well collecting plate.

Save previous collecting plate to use as a humidifier plate in step 38.

36. Add Trypsin Working Solution to all wells containing gel slices.
For 1–2 slices/well, add 30 μ l/well trypsin working solution. For 3 gel slices/well, add 45 μ l/well trypsin working solution.
37. Assemble plates with addition of a humidifier plate to prevent sample evaporation as shown in Figure 6. Incubate for 4 hr at 37° C in a “dry” incubator with circulating heated air such as the Taitec M-36 Microincubator, followed by cooling for 15 min at RT.
38. Disassemble the plates, leaving only the pierced plate, collecting plate, and polystyrene plate cover.
39. Centrifuge plates for 1 min at 300 $\times$ g, RT, to collect 1st extract into collecting plate.
40. Add 25 μ l Digest Extraction Buffer to wells, and incubate for 30 min at 37 °C followed by cooling for 15 min at RT.
41. Centrifuge plates for 1 min at 300 $\times$ g, RT, to collect 2nd extract into collecting plate with 1st extract.
42. Add 20 μ l/well Acetonitrile Extraction Buffer, and incubate for 15 min at 37 °C.
43. Centrifuge plates for 1 min at 300 $\times$ g, RT, to collect 3rd extract into collecting plate with 1st and 2nd extracts.
44. Transfer extracts into autosampler tubes.

Pool extracts from digestion wells that correspond to the same fraction from the same sample; e.g. if 3 replicate gel lanes were run for a sample, and corresponding slices were not combined prior to digestion, combine the 3 wells containing the same fraction at this step. Alternatively, digests from adjacent fractions in a single lane (e.g. fractions 1–2, 3–4, 5–6, etc.) may be pooled to reduce the number of LC-MS/MS runs (see Strategic Planning).

If running 0.5 cm gels (Alternative Protocol), combine digests corresponding to the same sample.
45. Flash freeze samples in dry ice/ethanol or liquid nitrogen.
46. Dry samples in a SpeedVac™ centrifuge, and store at –20 °C until ready for LC-MS/MS analysis.
47. Store plates containing digested gel slices at 4 °C.

Typically, these digested gel slices are not further analyzed, but it might be desirable to re-extract the digested gel slices in rare cases.

LC-MS/MS Analysis

48. Re-dissolve samples in Digest Resuspension Buffer for analysis by LC-MS/MS.

Volume of buffer to use should be determined by estimating the total amount of peptides in the sample and the amount of peptides that should be injected. For 75

µm i.d. LC columns, an appropriate load is about 1.0 µg of peptides on column for very complex mixtures such as cell lysates. This can be estimated by dividing the amount of total protein applied to the digested gel lanes divided by the number of fractions per lane.

49. If needed, digested samples can be cleaned up prior to LC-MS/MS using a C18 cartridge or equivalent.

We typically use a nanoLC with in-line reversed-phase trap column (e.g. Waters, catalog #186006527). Loading the sample onto the trap column followed by a several volume wash of the trap column removes most salts, buffer, and reaction byproducts. This is preferred rather than manually cleaning up samples prior to LC-MS/MS analysis because it is automated and more reproducible than manual offline cleanup methods. Also, the reagents used in this in-gel digestion protocol do not typically introduce unacceptable background during MS analysis.

If analyzing single fraction proteomes from 0.5 cm gels, go to Alternative Protocol, step 11. Otherwise, continue to next step.

50. Perform LC-MS/MS analysis.

Reversed-phase analytical separation is performed using a C18 column (e.g. Waters, catalog #186003546). Solvent A is 0.1% formic acid in Milli-Q® water, and Solvent B is 0.1% formic acid in acetonitrile. Fractions are run on a 70 min gradient with solvent B: 5–28% B over 60 min, 28–40% B over 5 min, 40–90% B over 1 min, and constant 90% B for 4 min. Column is re-equilibrated at initial conditions for 10 min. Potential carryover between samples can be minimized by injecting solvent A and using a 30 min gradient (5–90% B) with the same solvents between experimental runs (blank run).

51. Analyze by data-dependent MS/MS.

Analyses should be performed using a Q Exactive HF mass spectrometer or similar high-resolution, accurate-mass instrument. Appropriate MS/MS parameters are somewhat instrument dependent. For label-free analysis using a Q Exactive HF, full MS scans are acquired in profile mode at 60,000 resolution with a 400–2000 m/z scan range. Data-dependent MS/MS is performed on the top 20 most abundant precursor ions in every full MS scan. Unassigned and +1 charge ions are rejected, and “peptide match” is set to “preferred”.

Data Analysis

52. Analyze data from LC-MS/MS runs using a proteomics software suite, such as MaxQuant (Cox & Mann, 2008) or Proteome Discoverer (ThermoScientific).

Database searches should be performed using an appropriate species database with an appended contaminants database containing common contaminants such as keratins, trypsin, and likely sample specific contaminants, such as bovine serum proteins for cells grown in culture using fetal calf serum. Note that when cultured samples are being analyzed, the contaminants database should include

mycoplasma sequences as mycoplasma is a frequently unexpected contaminant in cultured cells. A reverse decoy database is used to control false discovery rate (FDR). For modern instrumentation, an FDR < 1% is typically required for peptides and protein groups.

ALTERNATIVE PROTOCOL

1D SDS GEL CLEANUP AND IN-GEL TRYPSIN DIGESTION FOR 1-SHOT PROTEOME ANALYSIS

Fractionation prior to LC-MS/MS analysis may not be required to achieve the targeted depth of analysis for certain samples and applications. However, 1D SDS gels can be used in this situation as a convenient sample cleanup method, particularly for samples with low amounts of total protein or those that contain MS-incompatible detergents, large amounts of non-volatile salts or other impurities that can interfere with either trypsin digestion or LC-MS/MS analysis. In this protocol, samples are run using 1D SDS gels for 0.5 cm. The gel is then fixed and stained. The entire region containing proteins (Figure 4B) is excised, reduced, alkylated, and digested by trypsin in-gel prior to LC-MS/MS analysis. Sample processing is otherwise like the Basic Protocol. A general overview of the protocol is shown in Figure 1.

1D SDS Gel Electrophoresis

1. Use a permanent marker to make a mark 0.5 cm from the bottom of sample wells on a 10% Bis-Tris, 1.0 mm, 15-well NuPAGE[®] gel cassette.

15-well gels are preferred rather than 10-well gels for this application to minimize the gel volume that contains sample. However, if a larger sample loading volume (up to 20 µl/lane) is needed, 10-well gels can be used.

2. Add SDS Protein Solubilizing Buffer (5X) to samples to a final concentration of 1X.

The well volume of the 1.0 mm, 15-well NuPAGE[®] gel used in this protocol is 15 µl. Therefore, the maximum sample load is 12 µl plus 3 µl 5X Solubilizing Buffer.

3. Heat samples for 2 min at 90 °C.
4. Assemble XCell SureLock[™] Mini-Cell unit and add 1X MES Running Buffer to both chambers.
5. Load samples into lanes.

Molecular weight markers are less useful on very short gels because very little size separation occurs under these conditions.

6. Run gel apparatus at 200 V using constant voltage.
7. Stop when the dye front has migrated 0.5 cm (about 5 min).

Go to Basic Protocol step 8 through step 19.

8. Use a clean razor blade to excise entire stained region of each gel lane (see Figure 4B).
- Unlike in Figure 4A, do not trim away the sides of the lane. Also, do not remove the very top of the gel lane as large proteins will have barely entered the gel under these conditions.
- Cut gel on top of lightbox covered with glass plate. Rinse working surface and cutting tools with methanol followed by Milli-Q® water prior to cutting. Do not allow methanol to contact the gel as that will result in gel dehydration.
9. Cut each excised lane vertically into 1.0 mm x 5.0 mm gel slices (see Figure 4B). A 15-well gel lane yields 4 gel slices, while a 10-well gel lane yields 6 gel slices.
10. Using forceps, transfer gel slices to individual wells of a 96-well pierced plate that has been stacked on top of a V-bottomed 96-well collecting plate.
- Split gel slices for each excised gel lane equally among 2 wells, i.e., place two gel slices per well from a 15-well gel lane or three slices per well from a 10-well gel. A maximum of three gel slices is recommended for a single well due to increased possibility of peptide trapping in larger gel volumes.
- Go to Basic Protocol step 22 to step 49.
11. Perform LC-MS/MS analysis.
- Reversed-phase analytical separation is performed using a C18 column (e.g. Waters, catalog #186003546). Solvent A is 0.1% formic acid in Milli-Q® water, and Solvent B is 0.1% formic acid in acetonitrile. Samples are run on a 4 hr gradient as follows: 5–30% B over 225 min, 30–80% B over 5 min, and constant 80% B for 10 min. Column is re-equilibrated at initial conditions for 10 min. Potential carryover between samples can be minimized by injecting solvent A and using a 30 min gradient (5–85% B) with the same solvents between experimental runs (blank run).
- Go to Basic Protocol step 51 to step 52.

SUPPORT PROTOCOL

REDUCTION AND ALKYLATION PRIOR TO 1D SDS GEL ELECTROPHORESIS

Reducing and alkylating samples prior to running gels saves time during the in-gel digestion protocol, especially for large-scale experiments with many fractions and/or samples. A convenient way to prepare these samples is to either dry samples in a SpeedVac™ centrifuge or precipitate the protein solution using 9 volumes of 100% ethanol or acetone followed by evaporation of residual solvent. However, there are several important considerations before using either of these methods. First, it is necessary to carry out ethanol or acetone precipitations at very low temperatures (–20 °C or lower) to ensure efficient precipitation. Also, there may be large and variable losses at this step, especially if the protein concentration is low or if the pH of the sample is extremely acidic or basic. Therefore, it is best to work at a neutral pH. If samples need to be dried, they should preferably be in a

volatile buffer, such as ammonium bicarbonate. The following protocol assumes that a protein sample has been dried in a SpeedVac™ or precipitated using an organic solvent. If it is not practical to precipitate or dry samples prior to 1D SDS gel electrophoresis, samples may still be reduced and alkylated in-solution without prior cleanup. However, samples need to be effectively denatured and at pH 8.0–8.5 for optimal alkylation and cannot contain reagents that will react with the alkylating reagent. Also, the increased volumes from adding the denaturing, reducing, and alkylating reagents need to be considered and final concentrations should be adjusted accordingly.

Additional Materials (see Basic Protocol)—SDS Resuspension Buffer: 1.0% (w/v) SDS in 100 mM Tris-HCl, pH 8.0 (see recipe in Reagents and Solutions)

1 M dithiothreitol (DTT) (see recipe in Reagents and Solutions)

0.5 M iodoacetamide (IAM) in 100 mM Tris-HCl, pH 8.6 (see recipe in Reagents and Solutions)

1. Resuspend protein pellet in SDS Resuspension Buffer.
2. Reduce samples by adding 1 M DTT (Final concentration: 20 mM). Incubate for 1 hr in a 37 °C incubator with shaking.
3. Alkylate samples by adding 0.5 M IAM in 100 mM Tris-Cl, pH 8.6 (final concentration: 60 mM). Incubate for 1 hr in a 37°C incubator with shaking, in the dark.
Cover incubator with tinfoil during incubation to prevent degradation of IAM.
4. Quench reaction by adding 1 M DTT to samples (final concentration: 15 mM). Incubate for 15 min in a 37 °C incubator with shaking.
5. Go to Basic Protocol, Step 1, and complete the protocol from there.

REAGENTS AND SOLUTIONS

Use Milli-Q® water or equivalent when preparing buffers and reagents.

Plate Wash Buffer: 0.1% (v/v) trifluoroacetic acid, 50% (v/v) methanol—

Combine 500 µl trifluoroacetic acid (Sigma), 250 ml Optima™ Methanol (Thermo Fisher Scientific), and 250 ml water. *Store at room temperature. Prepare fresh once a month.*

SDS Protein Solubilizing Buffer (5X): 1 M sucrose, 15% (w/v) SDS, 312.5 mM Tris-HCl, 10 mM Na₂EDTA, 5% (v/v) 2-mercaptoethanol, 5% (v/v) saturated bromophenol blue, pH 6.9—

Stock solution (5X): Dissolve 85.5 g sucrose (Sigma), 37.5 g SDS (Bio-Rad), 9.5 g Tris (Bio-Rad), and 0.925 g Na₂EDTA (Sigma) in 200 ml water. Slight heating may be used to completely dissolve all chemicals. Adjust pH to 6.9 with concentrated HCl. Bring final volume to 250 ml with water. *Store aliquots of stock solution in glass vials at 4 °C. Solution is stable for 6 months.*

Working solution (5X): Solubilize a stored aliquot of stock solution by microwaving it for ~10s. To 2 ml stock solution, add 100 µl 2-mercaptoethanol (Bio-Rad) and 100 µl saturated aqueous bromophenol blue solution (Bio-Rad). *Store working solution at room temperature. Solution is stable for 2 weeks. After first week, add fresh 2-mercaptoethanol.*

MES Running Buffer (1X): 50 mM MES, 50 mM Tris Base, 0.1% (v/v) SDS, 1 mM EDTA, pH 7.3—Warm NuPAGE® 20X MES Running Buffer (Thermo Fisher Scientific) at 37 °C or briefly in microwave (~10 sec) to solubilize SDS. Prepare 1X stock by diluting 50 ml 20X stock in 950 ml water and mix thoroughly. *Prepare fresh on day of use.*

Fixing Solution: 50% (v/v) methanol, 10% (v/v) acetic acid—Combine 50 ml Optima™ Methanol (Thermo Fisher Scientific), 10 ml glacial acetic acid (Thermo Fisher Scientific), and 40 ml water. *Prepare fresh on day of use. Keep at room temperature.*

Novex® Colloidal Blue Staining Kit (Thermo Fisher Scientific) Staining Solution—Combine 20 ml Optima™ Methanol (Thermo Fisher Scientific), 20 ml Stainer A, and 55 ml water. *Prepare fresh on day of use. Final volume will be 100 ml upon addition of 5 ml Stainer B to gel container, as indicated. Keep at room temperature.*

0.4 M ammonium bicarbonate, pH 8.0—Dissolve 15.81 g ammonium bicarbonate (Sigma) in 500 ml water. pH should be approximately 8.0 without adjustment. Filter solution on 0.22 µm filter. *Store at 4 °C. Stable for 3 months.*

Destaining Solution: 0.2 M ammonium bicarbonate, 50% (v/v) acetonitrile—Combine 10 ml 0.4 M ammonium bicarbonate and 10 ml HPLC grade acetonitrile (Thermo Fisher Scientific). *Prepare fresh on day of use. Keep at room temperature.*

Reducing Solution: 20 mM tris(2-carboxyethyl) phosphine hydrochloride, 25 mM ammonium bicarbonate—Dissolve 0.198 g ammonium bicarbonate (Sigma) in 40 ml water. Dissolve 0.573 g tris(2-carboxyethyl) phosphine hydrochloride (Thermo Fisher Scientific) in ammonium bicarbonate solution. Adjust pH to 8.0 with 5 M NaOH. Bring final volume to 100 ml with water. Filter solution on 0.22 µm filter. *Store aliquots at -20 °C. Solution is stable for 6 months.*

Alkylating Solution: 40 mM iodoacetamide, 25 mM ammonium bicarbonate—Dissolve 0.198 g ammonium bicarbonate (Sigma) in 40 ml water. Dissolve 0.74 g iodoacetamide (Sigma) in ammonium bicarbonate solution. pH should be 8.0 ± 0.2. Bring final volume to 100 ml with water. Filter solution on 0.22 µm filter. *Iodoacetamide is light sensitive. Store aliquots in dark at -20 °C. Solution is stable for 6 months.*

Wash Buffer 1: 25 mM ammonium bicarbonate—Add 1 ml 0.4 M ammonium bicarbonate to 15 ml water. *Prepare fresh on day of use. Keep at room temperature.*

Wash Buffer 2: 25 mM ammonium bicarbonate, 50% (v/v) acetonitrile—Combine 1 ml 0.4 M ammonium bicarbonate, 7 ml water, and 8 ml HPLC grade acetonitrile (Thermo Fisher Scientific). *Prepare fresh on day of use. Keep at room temperature.*

Trypsin Stock Solution—Dissolve one vial Sequencing grade modified trypsin (Promega, #V5111) (20 µg) in 200 µl of Trypsin Resuspension Buffer. *Prepare solution on ice. If the entire vial of trypsin is not needed, aliquot and freeze 50 µl (5 µg) aliquots and store at –20 °C. Solution is stable for 3 months.*

Trypsin Working Solution: 4 ng/µl trypsin, 40 mM ammonium bicarbonate—To 50 µl of Trypsin Stock Solution, add 1,075 µl Milli-Q® water and 125 µl 0.4 M ammonium bicarbonate. *Prepare on ice immediately before use.*

Digest Extraction Buffer: 4.5% (v/v) formic acid, 40 mM ammonium bicarbonate—Combine 1 ml 0.4 M ammonium bicarbonate, 450 µl neat LC-MS grade formic acid (Thermo Fisher Scientific), and 8.55 ml water. *Prepare fresh on day of use. Keep at room temperature.*

Acetonitrile Extraction Buffer: 80% (v/v) acetonitrile—Combine 12 ml HPLC grade acetonitrile (Thermo Fisher Scientific) and 3 ml water. *Prepare fresh on day of use. Keep at room temperature.*

Digest Resuspension Buffer: 0.1% (v/v) formic acid, 3% (v/v) acetonitrile—Combine 10 µl neat LC-MS grade formic acid (Thermo Fisher Scientific) and 300 µl HPLC grade acetonitrile (Thermo Fisher Scientific). Bring to 10 ml with water. *Prepare fresh on day of use. Keep at room temperature.*

SDS Resuspension Buffer: 1% (w/v) SDS in 100 mM Tris-HCL, pH 8.0—Dissolve 0.606 g Tris (Bio-Rad) in 40 ml water. Dissolve 5g SDS (Bio-Rad) in Tris solution. Adjust pH to 8.0 with 1M HCL. Bring final volume to 50 ml with water. Filter solution on 0.22 µm filter. *Prepare fresh on day of use. Keep at room temperature.*

1 M dithiothreitol (DTT)—Dissolve 7.71 g dithiothreitol (GE Healthcare) in 50 ml of water. Filter solution on 0.22 µm filter. *Store aliquots at –20 °C. Solution is stable for 6 months.*

0.5 M iodoacetamide (IAM) in 100 mM Tris-HCL, pH 8.6 (light sensitive)—Dissolve 0.606 g Tris base (Bio-Rad) in 40 ml water. Dissolve 4.62 g iodoacetamide (Sigma) in Tris solution. Adjust pH to 8.6 with 1M HCL. Bring final volume to 50 ml with water. Filter solution on 0.22 µm filter. *Iodoacetamide is light sensitive. Store aliquots in dark at –20 °C. Solution is stable for 6 months.*

COMMENTARY

Background Information—In-gel digestion, originally described by Shevchenko et al. (Shevchenko, Wilm, Vorm, & Mann, 1996), is often used to prepare samples for bottom-up or shotgun proteomics as an alternative to in-solution digestion (Washburn, 2008) or filter-aided sample preparation (FASP) (Wisniewski, Zougman, Nagaraj, & Mann, 2009). A major advantage of the in-gel method is that SDS as well as salts and buffers that may interfere with downstream processing steps are removed during the gel electrophoresis/fixing/staining procedure with minimal protein losses. Also, SDS will typically extract and solubilize more

proteins, particularly membrane proteins, than extractions using 8M urea or other in-solution digestion compatible reagents.

If SDS is used to solubilize proteins prior to in-solution digests, it must be removed via ethanol or acetone precipitation prior to the trypsin digestion step. However, precipitation to remove SDS can result in substantial and variable losses, particularly when working with low amounts of protein. Losses can occur due to incomplete precipitation at low protein concentrations, accidental loss of precipitate when removing the supernatant, and adsorptive losses after removing the SDS. While adsorptive losses can be reduced by using low protein binding microfuge tubes for in-solution digests, the other sample loss factors remain problematic. As illustrated in Figure 7A, ethanol precipitation of 25 μ g cell lysates showed consistent recoveries (lane 3–5) but recoveries from replicate 5 μ g aliquots were highly variable (lanes 6–8). In addition, when low levels of proteins are digested in solution, adsorptive losses to plastic and glass surfaces are more of a problem, even when low protein binding plasticware is used. As a result, similar numbers of proteins are identified when either 5 or 25 μ g of cell lysates are digested in-gel, whereas in-solution digests show a slight reduction in the number of identified proteins at the 25 μ g level and much lower, more variable yields at the 5 μ g level (Figure 7B).

As discussed in Strategic Planning, in-solution digestion is more practical than in-gel digestion for protocols that require a large amount of initial sample, e.g. 10–20 mg for enrichment of post-translationally modified peptides (Zhong, Molina, & Pandey, 2007). FASP is a middle ground between in-gel and in-solution approaches because it uses proteins solubilized in SDS followed by buffer exchange using ultrafiltration membranes into urea prior to trypsin digestion. Observed losses in FASP are typically around 50% and may depend on the specific filtration unit (Wisniewski, Zielinska, & Mann, 2011). Also, the many ultrafiltration steps are time consuming, and filter membranes may be damaged during processing leading to complete sample loss. Efforts to increase yield and reduce variability of FASP involve use of mild detergents and surfactants (Erde, Loo, & Loo, 2017).

In addition to the technical advantages mentioned above, running samples for a short-distance (2–4 cm) on a gel allows valuable visualization of the protein sample to be analyzed by LC-MS/MS. For example, colorimetric densitometry can be used to verify protein amounts and the gel pattern provides an assessment of sample quality prior to LC-MS/MS analysis (Paulo, 2016). Furthermore, fractionation of intact proteins by 1D SDS gel electrophoresis preserves information about protein molecular weight and can provide some insights into possible protein processing, major post-translational modifications, and alternative isoforms that may be missed by other fractionation procedures (Steen & Mann, 2004). This knowledge of the precise molecular form(s) of a protein that is associated with a disease or medical condition can be particularly useful for designing downstream targeted validation assays such as multiple reaction monitoring (MRM) (Beer, Tang, Sriswasdi, Barnhart, & Speicher, 2011). Finally, protein samples can be stored long-term once run on a gel without degradation; that is, stained and destained gels can be stored for at least one year at 4 °C in a Ziploc® bag without noticeable sample loss or deterioration.

Critical Parameters—Gel type, running buffer formulation, and gel stain are important factors to consider for Gel-LC-MS/MS analyses. Pre-cast, commercially available acrylamide gels such as NuPAGE® (Thermo Fisher Scientific) save time and are highly reproducible. A 10% or 12% Bis-Tris gel combined with NuPAGE® MES running buffer can effectively separate complex samples and provide resolution of high and medium molecular weight proteins, even when run for 2 to 4 cm. If resolution of the lower molecular weight region is preferred, NuPAGE® MOPS buffer can be used. It is not recommended to use gels with acrylamide percentages of 7% or lower because these gels, especially the first centimeter that contains an integrated stacker, are very soft and can be difficult to cut into uniform slices. Gel thickness and number of wells are also important considerations; 1 mm thick gels are optimal for peptide recoveries after in-gel digests (Speicher, Kolbas, Harper, & Speicher, 2000). Also, 10-well gels allow larger volumes to be loaded and are ideal for gel fractionation because lane edge effects can be removed by trimming the sides with a razor blade while retaining an ~4 mm wide gel slice on which the digestion solution volumes have been based. For single fraction proteomes, 15-well gels are preferred because more samples can be loaded per gel, and the decreased gel volume in the narrower lanes increases peptide recoveries.

When running more than one gel when fractionation will be used and samples will be quantitatively compared using label-free quantitation (LFQ), it is critical to measure the separation distances precisely to minimize gel-to gel migration variability. We typically run gels in separate electrophoresis chambers because front and back gels in the same electrophoresis unit can migrate differently. Therefore, it may be difficult to achieve reproducible gel separation in a standard gel chamber (e.g. XCell SureLock™ Mini-Cell, Thermo Fisher Scientific). The Mini Gel Tank available from Thermo Fisher Scientific can accommodate up to two gels in a convenient side-by-side format where migration between gels is more uniform.

When choosing a gel stain, the most important considerations are sensitivity and compatibility with mass spectrometry. This protocol describes gels stained with a commercial colloidal Coomassie reagent. In general, colloidal stains are rapid, require simple preparation, and are 5- to 10-fold more sensitive than conventional Coomassie Brilliant Blue stains. Silver stains are the most sensitive protein stains available; however, even MS-compatible silver stains reduce MS signals somewhat and the associated in gel digestion procedure is more time consuming. However, under staining is usually not a critical issue in these protocols because the entire region of the gel that contains proteins is digested. For a more complete list of protein gel stains and their compatibility with mass spectrometry refer to (Beer & Speicher, 2018).

As with other bottom-up sample preparation methods, trypsin is the protease of choice for most applications. This is because digestion is highly specific for the C-terminal side of lysine and arginine residues in proteins (Vandermarliere, Mueller, & Martens, 2013). Also, trypsin yields multiple peptides from most proteins that are in the ideal size range for LC-MS/MS analysis (7–25 residues) and these peptides typically have charge states from +2 to +4, which are well fragmented in the mass spectrometer. Also, the positioning of strong positive charges at the N- and C-termini of tryptic peptides typically produces fairly

complete complementary fragment ions. Alternative proteases may be used in combination with or instead of trypsin to increase sequence coverage. One approach for dealing with difficult to digest samples is to first digest with LysC, a protease that cleaves on the C-terminal side of lysine residues, then digest with trypsin. In other cases, digestion may be performed using exclusively alternative proteases with differing substrate specificities, e.g. LysC, AspN, GluC, ArgC, LysN, and chymotrypsin. Refer to the reference by Washburn for a comprehensive discussion of proteases (Washburn, 2008).

In-gel digestion conditions for trypsin and alternative proteases need to be carefully considered. We evaluated the effects of sample amount per lane (5 or 25 µg) using 0.5 cm electrophoresis, trypsin amount (4 or 20 ng), and trypsin incubation duration at 37 °C (4 or 20 hr) on the number of identified peptides, missed cleavages of tryptic sites, and identified proteins (Figure 8). For 4 hr digests, low levels of trypsin led to more missed cleavages than high levels of trypsin, regardless of starting material amount; however, there was a minimal effect on overall peptide and protein identifications. Extending the digestion duration from 4 hr to 20 hr resulted in lower peptide and protein identification, consistent with a substantial decrease in missed cleavages. This is primarily because a moderate degree of incomplete cleavage is desirable as it improves identification of sequences with a high density of tryptic sites that would otherwise produce short peptides (<7 residues) that are not identified in normal workflows. The lower trypsin level may also reduce ion suppression in the MS scan. From these results, digestion with a low amount of trypsin (4 ng) for a short duration (4 hr) works well for typical discovery proteomics while reducing both the time and cost of in-gel digests. Samples that are difficult to digest (e.g. cross-linked samples) or that will be used in targeted MS analyses where consistent digestion is critical (e.g. MRM) should be incubated overnight (20 hr) with the higher trypsin level (20 ng) to ensure endpoint digestion.

Moderate sample loss occurs during in-gel digestions due to multiple factors (Speicher et al., 2000). First, losses occur during gel fixing and staining because some protein at the surface of the gel can diffuse out of the gel before fixation is complete. Second, insufficient extraction of peptides from the gel matrix can occur and this loss increases as gel volumes increase. Also, adsorptive losses of peptides after digestion to plastic tubes and pipette tips occurs and can be significant at low peptide concentrations. We have estimated that recoveries after in-gel digestion range from 50– 80% (Speicher et al., 2000) depending upon the above factors. An optimal load of a tryptic peptide digest from a very complex sample such as a cell lysate on a 75 µm C18 column interfaced with a modern mass spectrometer is on the order of 0.5 to 1.0 µg, although in some cases, greater depth of analysis can be achieved by injecting up to 2.0 µg of digest. The simplest method of estimating amount on column is to assume a 100% recovery based on the amount of sample applied to the gel and initially target injection of 1.0 µg onto column, although as noted above, this will be a moderate overestimation. A more conservative approach is to assume an overall 50% recovery relative to the amount applied to the gel.

A sample may be divided into multiple adjacent lanes if the required sample volume is greater than the maximum capacity of a gel well or if the necessary protein amount per fraction would overload a gel lane. Corresponding slices from multiple lanes may be combined in a single trypsin digestion reaction or can be combined post-digestion as

described above. Quantitation of sample amounts using conventional protein assays prior to 1D SDS gel electrophoresis may not be practical or may be inaccurate due to interferences. For these samples, a strategy to estimate amount on the gel is to include a series of standards with known concentration (e.g. *E. coli* lysates) on the gel (see Figure 4A, lanes 2–4). The stained gel is then scanned and densitometry of stain for the entire lanes are calculated using software such as ImageJ (Schneider, Rasband, & Eliceiri, 2012) to estimate total protein amount in the gel. This method works reasonably well as long as the protein load does not greatly exceed the linearity range of the stain and the standard sample covers the same range as the samples.

Troubleshooting—A key issue with processing samples in 96-well plates (Basic Protocol) pertains to the pierced plate used for the digestion. These plates must have openings that allow for efficient removal of reagents using low speed centrifugation but also allow for sufficient surface tension to retain solutions during incubation steps throughout the protocol. We extensively used custom pierced plates with $3\text{--}5 \times 10 \mu\text{m}$ openings that worked quite well but are no longer available. We have also used suitable protein digestion plates that were commercially available from 4titude® but have been discontinued. There are currently several commercial pierced plates available that are marketed as in-gel digest compatible (from LVL Technologies and GlySci), although they have not been tested by us. Any pierced plates purchased for use in this protocol need to be tested to ensure that they will not leak the reagents used until centrifugal force is applied. In this regard, it should be noted that the presence of an organic solvent such as those with 50% or 80% acetonitrile, as used in the Basic Protocol, greatly reduce surface tension. However, some leakage under static conditions when using these reagents is not a major problem provided an appropriate collection plate is used during these steps. Hence, any pierced collection plate that will retain totally aqueous solutions can be used successfully.

A general concern that pertains to proteome analyses is contamination of samples with skin and hair keratins that are abundant in laboratory airborne “dust”. Precautions that should reduce keratin contamination to acceptable levels include: 1) perform all trypsin digestion steps in a laminar PCR hood using a polyethylene gown and nitrile gloves (note: latex gloves may contain keratin and other protein contaminants); 2) thoroughly clean gel running apparatus with detergent, rinse with Milli-Q® water, then protection from airborne dust; and 3) all working surfaces and tools used in gel processing should be rinsed with methanol followed by Milli-Q® water before initial use and between samples. Furthermore, plastics that contact samples, including pierced plates and autosampler tubes, should be prewashed with 0.1% trifluoroacetic acid and 50% methanol to reduce polymers that can be observed in MS spectra. Milli-Q® water or equivalent should be used when preparing all buffers and reagents. Complete elimination of contaminants is nearly impossible; therefore, even with the above precautions, it is necessary to account for keratins and other common contaminants in the search database to reduce false positive identifications.

Anticipated Results—In our experience, approximately 8,000 proteins can be identified in a cancer cell proteome with 10 fractions, compared to about 5,000 proteins in a single fraction, when run time is fixed at 4 hr (Figure 2B). However, this increased depth of

analysis comes at the cost of extensive instrument time per sample, and there is a clear non-linear relationship between protein identifications and analysis time. For example, when cell lysates were analyzed, there was only a 5% gain in protein identifications between 5 and 10 fraction proteomes despite doubling instrument time from 20 to 40 hr. However, this large investment of instrument time can be substantially reduced by using shorter gradients for analysis of fractions. In our experience, 4 hr gradients are optimal for one-shot (no fractionation) proteomes, while 70 or 90 min gradients are the best compromise between depth of analysis and total instrument time when 5 or more fractions are analyzed per proteome (Figure 2A). Depth of analysis for biological fluids follows similar trends but overall depth of analysis is much lower for similar investments of instrument time due the wider dynamic range of protein abundances in biological fluids compared with cell lysates. For example, LC-MS/MS analysis of major protein depleted human plasma can identify approximately 450 proteins in a single 70 min gradient, 500 proteins in a 90 min gradient, and 700 proteins in a 4 hr gradient (Figure 3A). Fractionation into 2, 4, or 8 fractions identifies 700, 900, or 1,200 proteins, respectively, when each fraction is analyzed in a 90 min gradient (Figure 3B).

The total numbers of proteins identified is also influenced by the quantitation method used. A common method is label-free quantitation (LFQ), which integrates the ion area of peptide peaks in MS1 spectra and sums areas of all peptides matched to a protein. An interesting feature of LFQ is that the total number of peptides and proteins identified in an experiment increases with the number of samples. This is because selection of weak signals for MS2 analysis is somewhat stochastic and not all weak MS2 result in a positive identification. However, “matching between runs” transfers peptide identifications across samples based on accurate mass and retention time even if a peptide identification did not occur in a single sample. This results in much greater identification of peptides and proteins using LFQ, which is supported by MaxQuant and other proteomics software packages (Geiger, Wehner, Schaab, Cox, & Mann, 2012).

Time Considerations—Sample preparation typically requires 3–4 days of operator time as follows:

- Day 1: Setting up the gel apparatus and preparing buffers takes ~20 min. Preparing samples and loading the gel takes ~30 min. For 1D SDS gel electrophoresis, a 2 cm gel takes ~15 min to run and a 0.5 cm gel takes ~5 min to run. Fixing the gel takes ~10 min. Staining the gel with colloidal Coomassie takes 3 hr to overnight.
- Day 2: Destaining the gel takes overnight but can be shortened with repeated exchanges of fresh Milli-Q® water.
- Day 3: Processing the gel and reduction/alkylation takes about one day.
- Day 4: Digesting with trypsin, eluting peptides, and drying samples take ~1 day.

If necessary, the steps described on Day 1 and Day 2 can be combined if gels are stained with colloidal Coomassie for 3 hours rather than overnight. Mass spectrometer time depends on the number of samples, extent of fractionation, and gradient length (Figure 2 and 3). For

example, a sample with 10 fractions each analyzed using a 70 min gradient would require approximately 19 hr of instrument time including overhead of re-equilibration and blank runs. A sample analyzed as a single fraction using a 4 hr gradient would require 5 hr of instrument time.

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SIGNIFICANCE

One-dimensional sodium dodecyl sulfate (1D SDS) gels represent a simple method for either cleaning up proteome samples or fractionating proteomes prior to proteolysis and subsequent nanocapillary HPLC coupled to tandem mass spectrometry analysis (LC-MS/MS). Because 1D SDS gels are used in most biological research laboratories, they are a convenient sample processing method that is appropriate for many, but not all, proteome studies that use “shotgun” or “bottom-up” proteome analysis by LC-MS/MS (see Strategic Planning). This method is frequently referred to as GeLC-MS/MS or Gel-LC-MS/MS.

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Figure 1.
Major steps in Gel-LC-MS/MS workflow and their relationships to the protocols.

Figure 2.

Protein identifications in cancer cell lines are dependent on LC gradient length and degree of sample fractionation using Gel-LC-MS/MS. Total number of proteins (protein and peptide false discovery rate of 1%) identified from cell lysates are shown. **A.** Protein identifications from single fraction and ten fraction proteomes of melanoma cell line 1205Lu. The unfractionated sample was run on a 4 hr gradient, and fractionated samples (10 fractions) were each run on 90 min gradients. **B.** Effect of fraction numbers (1, 2, 5, or 10 fractions) on protein identifications in ovarian cancer cell line OV90. All fractions were analyzed using a 4 hr LC gradient.

Figure 3.

Plasma protein identifications are dependent on LC gradient length and degree of sample fractionation. Total number of high confidence proteins identified (2 or more peptides identified, protein and peptide false discovery rates of 1%) from major protein-depleted plasma samples are shown. **A.** Effect of gradient length on number of proteins identified in a single fraction. Three gradient times (70 min, 90 min, and 4 hr) were evaluated. **B.** Effect of fraction numbers (1, 2, 4, or 8 fractions) on protein identifications. All fractions were analyzed using a 90 min LC gradient.

Figure 4.

Representative samples for proteome analysis on 1D SDS mini gels. **A.** In-gel fractionation after electrophoretic separation for 2 cm. Left Panel: 10-well 10% Bis-Tris NuPAGE® gel. Lane 1: MW Std. Lanes 2–4: *E. coli* standard representing 30, 10, and 3.3 µg of protein, respectively. Lanes 5–10: samples to be digested. Right Panel: Gel lanes are sliced vertically as shown to remove the outer edge of the lane where vertical streaking commonly occurs. The central part of the lane is then sliced into uniform 1 × 4 mm horizontal slices. **B.** Sample cleanup without fractionation using a 0.5 cm electrophoretic fractionation. Left Panel: 15-

well 10% Bis-Tris NuPAGE® gel. Lane 1: MW Standard; Lanes 2–3: *E. coli* standard representing 9 and 3 ug of protein; Lanes 4–15: protein samples to be digested. Right panel: each vertical slice encompasses the entire region of electrophoretic separation.

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Figure 5. Razor blade array. **A.** Top view of razor blade array assembled with ~50 razor blades and an equal number of 1 mm Teflon spacers. **B.** Side view showing razor blades separated by 1 mm Teflon spacers. All metal components are stainless steel.

Figure 6.

Assembly of 96-well plates to reduce sample evaporation during tryptic digestions. Place pierced plate containing dried gel slices on top of clean collecting plate. Fill the wells of a collecting plate that was used during previous steps with 80 μ l water per well to create a humidifier plate. Stack pierced plate/collecting plate on top of humidifier plate as shown. Cover pierced plate with a fitted glass plate to minimize evaporation, and place a polystyrene plate cover over glass plate to complete the plate assembly. Before incubation at 37 °C, place the assembly into two sealed Ziploc[®] bags in opposite orientations. This creates an environment with high humidity that will prevent sample evaporation during the trypsin digestion.

Figure 7.

Comparison of protein identification depth using in-gel and in-solution digestion. OVCAR3 ovarian cancer cells were lysed using a Tris-SDS buffer. For in-gel digestion, aliquots of 5 µg and 25 µg were loaded directly to SDS gels, separated 0.5 cm, and digested using the Alternative Protocol (gel not shown). For in-solution digestion, additional replicate aliquots of 5 and 25 µg were precipitated using ethanol. **A.** Recovery of cell lysate after ethanol precipitation to remove SDS. The equivalent of 1 µg cell lysate assuming complete recovery was loaded per lane, and the gel was silver stained. Lane 1: MW Std. Lane 2: cell lysate before ethanol precipitation. Lanes 3–8: Protein recovered from 3 replicates of 25 µg (lanes 3–5) or 5 µg (lanes 6–8) cell lysate after ethanol precipitation. **B.** Protein identifications from in-gel and in-solution digests (same samples shown in panel A) of 5 µg or 25 µg OVCAR3 cell lysate. Total number of proteins identified (protein and peptide false discovery rate of 1%) are shown. All samples were analyzed using a 4 hr gradient. n=2 for in-gel digestions; n=3 for in-solution digestions. Error bars represent standard deviations.

Figure 8.

Effects of trypsin digestion conditions on depth of protein identification. Either 5 or 25 μ g of cell lysate were separated for 0.5 cm on a gel followed by digestion with either 4 or 40 ng trypsin per digest and with digestion for either 4 or 20 hr at 37 °C. Data shown are: **A**. Number of unique peptides identified; **B**. Breakdown of peptides based on number of missed cleavages; and **C**. Number of identified proteins. Data analysis was performed using MaxQuant with protein and peptide false discovery rate of 1%.