Chapter 1

A Robust Protocol for Protein Extraction and Digestion

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Abstract

Proteins play a key role in all aspects of cellular homeostasis. Proteomics, the large-scale study of proteins, provides in-depth data on protein properties, including abundances and post-translational modification states, and as such provides a rich avenue for the investigation of biological and disease processes. While proteomic tools such as mass spectrometry have enabled exquisitely sensitive sample analysis, sample preparation remains a critical unstandardized variable that can have a significant impact on downstream data readouts. Consistency in sample preparation and handling is therefore paramount in the collection and analysis of proteomic data.

Here we describe methods for performing protein extraction from cell culture or tissues, digesting the isolated protein into peptides via in-solution enzymatic digest, and peptide cleanup with final preparations for analysis via liquid chromatography-mass spectrometry. These protocols have been optimized and standardized for maximum consistency and maintenance of sample integrity.

Key words Proteomics, Protein extraction, Acetone precipitation, Enzymatic solution digest, Liquid chromatography-mass spectrometry

1 Introduction

Analysis of proteins, the key effectors of most cellular processes, is critical for understanding biological processes in healthy and diseased states, and liquid chromatography-mass spectrometry (LC-MS) analysis of peptides has proven to be a workhorse tool to this end. Over the last 10 years, LC-MS instrumentation for biological proteomics has undergone a remarkable evolution with significant gains in analysis speed, precision, consistency, and sensitivity and has in many cases given rise to commoditized instrumentation accessible even to non-experts [1]. As a result, a major factor in the success of proteomics-focused assays has now become the quality of the input material and the consistency of, and care in performing, methods to generate proteomic samples for LC-MS assays.

While the relative simplicity of nucleic acid analytes enabled a reasonably rapid and facile unification of methods in the genomics field, the huge variation in biophysical properties of proteins, and

Lucio Comai et al. (eds.), *Proteomics: Methods and Protocols*, Methods in Molecular Biology, vol. 1550,
DOI 10.1007/978-1-4939-6747-6_1,

their several orders of magnitude abundance variation in cells, has proven a significant challenge to the proteomics field [1]. Variations in methods for proteomics sample generation across labs, and even within groups across operators, can significantly affect and even confound the outputs of MS-based proteomics. This will undoubtedly prove to be a challenge going forward for the field in highly specific experimental contexts and in answering unique biological questions, especially given the highly varied biophysical properties exhibited by proteins and protein complexes. However, methods striving for complete solubilization of proteomes, for example for global profiling under different states or conditions, are much more amenable to unification across the field.

However, an operator striving to develop such a sample workup regime for global proteomics analysis faces what is now a daunting variety of choices at each step of the workflow. Often reagents for upstream sample preparation are not compatible with LC-MS analysis downstream, necessitating additional steps that can potentially cause sample loss. For example, while robust detergents are generally required to affect protein solubility, many common detergents used in this role, such as SDS, are not compatible with downstream proteolytic digestion and/or LC-MS. A wide variety of choices to deal with this challenge now face the operator including traditional methods such as protein precipitation for detergent removal [2], commercial innovations such as alternative LC-MS compatible surfactants (e.g., Rapigest SF Surfactant, Waters Corporation), and development of relatively new methods such as FASP (Filter-Aided Sample Preparation), a method facilitating detergent removal and digestion on a solid phase [3], just to name a few. In developing a composite workflow, assessing which of these approaches best balances robustness and consistency while avoiding significant sample loss is a difficult task. Moreover, a similarly daunting set of choices must be addressed at all other steps of a proteomics workflow, including a host of variables for proteolytic digestion and peptide cleanup, among others.

Here we provide a simple, robust method for whole-cell protein extraction from tissue culture cells, including methods for downstream enzymatic digestion and peptide cleanup prior to LC-MS. This method employs a classic ionic detergent at a relatively high percentage to efficiently extract and solubilize proteins. A facile acetone precipitation step is employed to remove this detergent prior to straightforward and proven in-solution proteolytic digestion. A simple solid-phase extraction method is performed at the end of the workflow to clean and concentrate peptides in advance of LC-MS. While no one method is perfect, based on our experiences working in the field we feel the simplicity and consistency of this method will provide new operators to the field with an accessible, robust, and consistent starting point, and in fact we use this routinely in our group for global proteomic analyses of our valuable primary samples. Specific method steps are

provided, and critical variables for each method are emphasized so that experimental bias is minimized as much as possible.

2 Materials

1. Cell Lysis and Acetone Precipitation.

Given the sensitivity of mass spectrometry analysis, utmost care should be taken to ensure clean working conditions free of all possible contaminants. All reagents should be prepared using LC-MS grade water (Honeywell brand is preferred). If possible, sequestering a separate set of pipettes along with the use of nitrile examination gloves is extremely useful in avoiding contamination of samples with ubiquitous keratin from the environment.

2.1 Solutions and Reagents

1. 4% w/v sodium dodecyl sulfate (SDS).
2. 1 M Tris hydrochloride, pH 7.5.
3. 1 M dithiothreitol (DTT): Dissolve 87 mg in 5 mL 100% MeOH, store at −20 °C.
4. Thermo HALT protease and phosphatase inhibitor cocktail (100×).
5. 1 M (10×) PMSF (phenyl-methyl-sulfonamide): Dissolve 87 mg in 5 mL LC-MS water, store at −20 °C.
6. LC-MS-grade water.
7. 99.5% + acetone, chilled to −20 °C.

2.2 Equipment

1. Probe tip sonicator (alternatively, a Diagenode or Covaris water bath sonicator, *see* **Note 1**).
2. (for probe tip sonicators) Clear plastic or acrylic box (6 × 6 × 6 in. works well).
3. (for probe tip sonicators) Foam tube floater.
4. Pierce Micro BCA Protein Assay Kit.

2.3 Peptide Digestion

2.3.1 Solutions and Reagents

1. 1 M TCEP: Dissolve 287 mg in 1 mL MS water. Store at −20 °C in suitable (e.g., 10 µL) aliquots.
2. 1 M Tris pH 8, *see* **Note 2**.
3. 500 mM iodoacetamide (IAA): Dissolve a single-use pre-weighed tube of 9.3 mg IAA (Pierce/Thermo Fisher) in 200 µL LC/MS water.
4. Protein resuspension solution: 8 M urea, 100 mM Tris pH 8.
5. Protein dilution solution: 100 mM Tris pH 8.
6. 100 mM $CaCl_2$.
7. Sequence-grade trypsin enzyme.

2.4 C18 Cleanup

2.4.1 Solutions and Reagents

1. Honeywell Brand Reverse Phase A (RP-A): LC-MS water with 0.1% formic acid.
2. Honeywell Brand Reverse Phase B (RP-B): acetonitrile with 0.1% formic acid.
3. Wetting solution (50% Honeywell RP-B in RP-A).
4. Equilibration/Wash solution (2% RP-B in Honeywell RP-A), *see* **Note 3**.
5. Elution solution (90% Honeywell RP-B in RP-A).

2.5 Equipment

1. Thermo Pierce C18 tips (Thermo product 87784).

3 Methods

3.1 Cell Lysis and Acetone Precipitation

1. Fill an ice bucket with wet ice and fill up to ice level with water.
2. Prepare 500 µL of cell lysis buffer for each 1e7 cells.
   (a) Lysis buffer composition: 3% SDS, 0.02 M DTT, 0.10 M Tris–HCl pH 7.5, 1× Thermo Protease/Phosphatase inhibitor, 1× PMSF. Make fresh and add protease/phosphatase inhibitors, especially PMSF that is active in aqueous solution for 30 min, just before use.
   (b) Preheat lysis buffer at 95 °C. *See* **Note 4**.
3. Keep cell pellet tubes on dry ice. Add preheated lysis buffer to frozen cell pellet; 500 µL for each ten million cells (*see* **Notes 5** and **6**). Pipette and/or vortex to mix. Critical point: the frozen pellet must not be allowed to thaw until covered with hot SDS lysis solution—thawing then should be rapid and right into the concentrated surfactant.
4. Put cap lock on tube. Place tube in 95 °C heat block for 3 min. Vortex every 15–20 s.
5. If necessary, split contents into tubes of ~500 µL each, *see* **Note 7**.
6. Pour chilled ice water from ice bucket into clear plastic/acrylic box (*see* **Note 8**), avoiding ice as much as possible. Wedge an ice pack to the bottom of the acrylic box to keep the water cool (*see* **Note 9**), and a foam tube holder to float the tubes at the surface.
7. Vent the tubes by opening them briefly (with the opening cap oriented away from you). Place them in the foam holder in the acrylic box.
8. Sonicate samples while the tubes remain in ice water (*see* **Note 10**). There are two critical points in this step, both facilitated by the use of the clear acrylic box: first, the samples must be kept cold to avoid degradation of proteins due to the heat generated by the sonicator. We have found that keeping

the tubes in water is the best way to accomplish this. Secondly, it is necessary to submerge the sonicator probe tip before switching on sonicator, and switch it off before removing the tip. Moving the probe in and out of solution while on will cause foaming of the solution and may damage proteins. Additionally, be consistent with tip depth (2/3 into solution from top is ideal).

(a) Set sonicator amplitude to 40%.

(b) Press the "Set" button to select "Continuous".

(c) Sonicate for 3 cycles. 1 cycle = ON for 30 s, OFF on ice for 2 min (*see* **Notes 11** and **12**). Avoid contact between probe tip and tube walls as much as possible to prevent shedding of polymers into your sample.

9. Centrifuge tubes for 15 min at 15,000 rpm at 20 °C to clarify the lysate of any particulates or insoluble material (*see* **Note 13**).
10. Transfer ~200–250 µL of supernatant to labeled Eppendorf tubes (*see* **Note 14**).
    (a) Pull out 10% of the volume for a separate precipitation to generate a pellet to bring up in 3% SDS for BCA quantitation (*see* **Note 15**).
    (b) Note the exact volume of each sample that goes into each new tube. This will be important later when calculating the amount of protein per tube.
11. Using a glass Pasteur pipette (*see* **Note 16**), add cold (−20 °C) 100% acetone to tubes. Fill to 1 mL line (*see* **Note 17**). Critical point: ensure that least 4× volume acetone is added.
12. Invert tubes several times, vortex well, and place tubes at −20 °C overnight (*see* **Note 18**).
13. The next day, spin tubes at 15,000 rpm for 15 min at 4 °C (*see* **Note 19**).
14. Keep tubes on ice. Without disturbing the pellet or pipetting up and down, remove and discard the acetone supernatant with a glass Pasteur pipette (*see* **Note 20**). Add fresh ice-cold acetone to the 1 mL mark, again without disturbing the pellet.
15. Centrifuge samples at 15,000 rpm for 10 min at 4 °C (*see* **Note 21**).
16. Repeat **steps 14** and **15** for a second wash (*see* **Note 22**).
17. Remove and discard acetone supernatant with a glass Pasteur pipette.
18. Air-dry pellets with the lids open (covered with a Kimwipes) for 1 h while on ice (*see* **Note 23**).
19. Store dry pellets at −80 °C until ready for further use.

20. Resuspend the pellet from the 10 % (v/v) aliquot precipitation from **step 10a** in 100–300 µL 3% SDS and perform BCA (*see* **Note 24**) quantification according to the manufacturer's instructions. From the results determine how much protein is in each acetone-precipitated pellet based on the fraction of the total sample that went into each tube. Critical point: ensure complete resuspension and solubilization of the pellet. In addition to visually inspecting the pellet to confirm complete resuspension, vortexing the sample and/or heating at 37 °C for up to 2 min can aid in resolubilization.

3.2 Peptide Digestion

1. Bring up dried acetone pellet in 50 µL 8 M urea, 100 mM Tris pH 8 for a final concentration of 80 µg in 25 µL (*see* **Note 25**).
   (a) Use manual pipetting and if necessary heat and/or sonication to break up pellet. Avoid any heating over 37 °C to avoid carbamylation by urea.
2. Add TCEP to final concentration of 5 mM and incubate for RT for 30 min (*see* **Note 26**). Mix well by vortexing, and then knock down by pulse spinning in microfuge.
3. Add IAA to final concentration of 10 mM and incubate for RT for 30 min in the dark (covered in foil, *see* **Note 27**). Mix well by vortexing, and then knock down by pulse spinning in microfuge.
4. Bring up to 250 µL with 100 mM Tris pH 8, reducing the urea concentration to <1 M (*see* **Note 28**). Mix well by vortexing, and then knock down by pulse spinning in microfuge.
5. Add 100 mM $CaCl_2$ to a final concentration of 1 mM. Mix well by vortexing, and then knock down by pulse spinning in microfuge.
6. Add 8 µg trypsin (1:10 mass:mass) for a final concentration of 30 ng/µL. Mix well by vortexing, and then knock down by pulse spinning in microfuge.
7. Incubate overnight at 37 °C in static or shaking incubator.

3.3 C18 Cleanup

The following is a modified protocol is based on the manufacturer's recommendations for Pierce C18 tips (100 µL bed, Catalog No. 87784).

Preloading tubes, or well plates, with each solution to be used can increase throughput speed and minimizes downtime in which the tip can dry. Each 100 µL aliquot of sample should be loaded separately, and should be separately aliquoted into wells or tubes.

1. Wet C18 filter tip by aspirating 100 µL of wetting solution and then discarding solvent (*see* **Note 29**).
2. Repeat wash step and discard solvent.
3. Equilibrate tip by aspirating 100 µL of equilibration/wash solution and discarding the solvent.

4. Repeat equilibration step and discard solvent.
5. Aspirate 100 µL of sample and, using a clean Eppendorf tube, slowly aspirate 100 µL up and down 10× (*see* **Note 30**).
   (a) Repeat as many times as necessary until the entire sample volume has been absorbed onto the column.
6. Wash tip by aspirating 100 µL of equilibration/wash solution and discarding the solvent.
7. Repeat the wash step and discard the solvent.
8. Elute the sample by slowly aspirating 100 µL of elution solution into the tip, and slowly depositing into a new elution tube.
9. Repeat the elution step for a total final sample volume of 200 µL. Deposit the second elution into the same tube as the first.
10. Dry the sample down to 10% of its elution volume in a SpeedVac (*see* **Note 31**).
11. Resuspend peptides in 0.1% formic acid in 2% RP-B in RP-A. Resuspend to a final volume of 1 µg/ul (or other suitable concentration and volume depending on your intended LC-MS injection amount).
12. Spin sample for 3 min at maximum speed in a centrifuge before transferring the supernatant to an autosampler vial for downstream mass spectrometry analysis (*see* **Note 32**).

4 Notes

1. The sonicator is used to shear the chromatin in the sample, decreasing its viscosity to allow for efficient protein recovery. Branson water baths are not powerful enough for this; only brands such as Diagenode and Covaris that are typically used for shearing fixed chromatin (e.g., for Chip-SEQ) will work, although the sonication efficiencies of water bath type instruments in our experience are much more sensitive to input amounts (e.g. input overloading) versus probe-tip sonicators.
2. Tris pH 8 is optimal when using trypsin as the digestive enzyme. If using LysC, substitute Tris pH 9 throughout the protocol.
3. Including a small amount of acetonitrile in the solution aids in solubilization of the peptides.
4. When heating solutions to near-boiling temperatures, it is helpful to add cap locks to the Eppendorf tubes to ensure that they stay closed. Furthermore, after boiling tubes should be opened facing away from you to vent and avoid splashing up of expanded air/liquid in the tube.
5. Scale volume of lysis buffer up for pellets with more cells.

6. Volume of lysis buffer stated is for probe tip sonicator; volumes should be adjusted for different sonicators according to manufacturer's recommendations. Additionally, appropriate tubes should be used if necessary —for example, older Diagenode models require their own commercial tubes which have a harder consistency for sufficient sonication efficiency.
7. 500 μL per tube is the ideal volume for sonication on a probe tip sonicator. Smaller volumes can be used with suitably powerful water bath sonicators.
8. For probe tip sonicators, it is essential to visually monitor the depth of the probe tip during sonication while keeping the sample cold. We recommend using a clear enclosure so that the placement depth of the probe tip can be visually ascertained for as much consistency as possible.
9. It is important to keep the samples as cold as possible to counteract the heating induced by sonication.
10. Invert tubes before and after sonication to check for viscosity—viscosity should be much reduced after sonication.
11. Different brands of sonicators have different strengths, so optimal conditions (where minimal sonication is applied to sufficiently disrupt chromatin) should be determined empirically for each sonicator. Slowly increasing strength and/or time of sonication and monitoring viscosity of the resulting lysate with test lysates (e.g., by inverting tubes and observing fluidity of the solution during a pilot timecourse) is useful for quickly determining an optimal sonication regime. Oversonication should be avoided to minimize heating and/or physical compromise of proteins in samples.
12. In the case of probe tip sonicators it is absolutely critical is to submerge the sonicator probe tip before switching on the sonicator, and switch it off before removing the tip.
13. Orient tubes inside centrifuge so hinge faces outward to consistently position the pellet.
14. Avoid touching pellet with pipette tip—do not hesitate to leave some supernatant behind if necessary. Split as necessary as you wan to be able to add at least 4–5 volumes of ice-cold acetone for a good precipitation.
15. A 10% precipitation is done to remove DTT and so that the lysate can be resuspended in a strong denaturant compatible with the protein quantification BCA assay. Interferences can significantly bias results of the assay.
16. It is important to always use glass when working with acetone—the alcohol can potentially solubilize plastic on pipette tips, causing polymer interference in downstream LC-MS analysis.

17. Again, make sure this is at least a fourfold volume addition of neat ice-cold acetone to ensure the dielectric constant of the solution is sufficiently reduced for efficient precipitation. Keep acetone on ice or put it back in the freezer when not in use.
18. Ideally should see some flecks of particulate matter starting to precipitate even before putting tubes into freezer, although visible precipitating material may not be immediately observable with inputs of less than 300 μg.
19. Again, orienting the tubes inside the centrifuge so that the hinges face outward will ensure you know where the pellet is.
20. Leaving behind a small amount (~10 μL) of acetone is preferable to disturbing the pellet.
21. The pellet will usually appear lightly opaque or white on the backside of conical bottom.
22. Here it is fine to simply add cold acetone and re-spin. No vortexing or release of pellet from tube required, or recommended.
23. Pellets may shrink during the drying process. Do not over-dry pellets, as this will make them more difficult to resuspend. Do not use the Speed-Vac at this step.
24. Run triplicates of each sample. May also run sample undiluted in addition to 1:5–1:20 dilutions to ensure accurate range of the BCA assay.
25. If sample amounts permit, reserve a small amount (equivalent of ~5 μg) for protein gel analysis pre- vs post-digest.
26. Dilute TCEP 1:10 in MS-grade water (e.g., dilute 1:10 and add 5 μL to each 100 μL digest).
27. Dilute single use tube (9.3 mg per tube) in 200 μL MS-grade water for 200 mM stock (e.g., add 5 μL to each 100 μL digest)—use of the pre-weighed tubes minimizes weighing errors and saves on reagent, which is not reusable once resuspended.
28. This dilution step is very important—trypsin is only active in up to 1 M urea. Less trypsin (to 1:50 w/w) can be used to reduce cost, but care should be taken to avoid incomplete digestion - trypsin concentration in the final volume should not drop below 20 ng/μL.
29. Once wet, do not introduce air into the tip resin—this could dry the column and cause any bound material to become permanently attached. This is important throughout the protocol, but especially after the methanol wetting step as methanol will evaporate quickly if delays occur. Likewise, proceed continuously through the protocol as any stopping or pauses could cause the tip to dry out.

30. Aspirations up and down into the tip should be done slowly (each single up/down cycle over 3–5 s, and multiple aspirations up and down into the tip can be done to improve efficiency. Recommended are 10 aspirations for each 100 μL aliquot of sample loaded, and 5 for each 100 μL elution, and 3 aspirations at every other step. With experience and care, implementation an 8-channel multichannel pipette and well plates can improve throughput speed.
31. This requires careful monitoring of the drying, which can be achieved with a strobe device (e.g. Labconco Centrizap) that allows visualization of the sample without stopping the Speed-Vac.
32. Avoid any pelleted material at bottom of tube. This final spin step helps remove any particulates or debris that may clog the mass spectrometer.

References

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