

Whole proteome analysis

This document describes an end-to-end protocol for quantitative whole-proteome analysis by mass spectrometry. This protocol is also appropriate for harvesting material for other assays, such as Western blotting, and does not require any specialized equipment (e.g. sonicator). This protocol can be used as a general guideline as there are many different ways of doing many of these individual steps and still achieving success. The topics covered include how to perform harvest and lysis of cell line material, clean-up of proteins, tryptic digestion, and final peptide clean-up. The protocol is designed to work with cell line material, but may be appropriate for tissues or other sources.

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1. RNA and Protein Sample Preparation

For all the solutions described below and throughout this protocol in general, you should do your best to use sterile glassware and reagents. As the goal here is to measure proteins, make an effort to minimize potential keratin contamination (e.g. wear a lab coat and don't touch your tubes with un-gloved hands).

1.1 Reagents and materials

- 1.5mL or 2.0mL Safe-Lock tubes (Thermo Scientific, CAT#05-402-25 or CAT#05-402-7)
- Benchtop centrifuge with holder for microcentrifuge tubes (multiple vendors)
- DPBS (Thermo Scientific, CAT#14190144)
- 1M TrisCl pH 7.5 (Thermo Scientific, CAT#J60636.K2)
- MgCl₂ (Sigma, CAT#M8266)
- KCl (Sigma, CAT#P9333)
- NP-40 (Sigma, CAT#NP40)
- Deoxycholate (DOC) (Sigma, CAT#D6750)
- 20% SDS (Thermo Scientific, CAT#BP1311)
- cOmplete protease inhibitor tablets, EDTA free (Sigma, CAT#11836170001)
- Dithiothreitol (Bio-Rad, CAT#1610611)
- Chloroacetamide (Sigma, CAT#C0267)
- Benzonase nuclease, 25U/uL (Sigma, CAT#70664-3)
- RNase A, 10mg/mL (Thermo Scientific, CAT#EN0531)
- Urea (Sigma, CAT#U5128)
- Clean water (Thermo Scientific, CAT#10977023)
- Trypsin/rLysC mix (Promega, CAT#V5071)
- Thermomixer with 2mL tube block (Eppendorf)
- Thermoblock set to >95C (multiple vendors)

- Cell scrapers (multiple vendors)
- Magnetic rack for 1.5mL or 2.0mL tubes (I like this rack: Promega, CAT#Z5342)
- Ethanol, absolute (whatever vendor sells this to your lab)
- SP3 beads (Sigma, CAT#65152105050250, CAT#45152105050250)
- Acetone (Sigma, CAT#179124)
- Strata-X microelution SPE plates (CAT# 8M-S100-4GA, Phenomenex)
- Methanol, HPLC grade (CAT#610090040, Thermo Scientific)
- Water, HPLC grade (CAT#51140, Thermo Scientific)
- Trifluoroacetic acid, HPLC grade (CAT#85183, Thermo Scientific)
- Formic acid, HPLC grade (CAT#85178, Thermo Scientific)
- 1.5mL snap-lock tubes (CAT#05-402-25, Thermo Scientific)
- Benchtop centrifuge with plate-holder rotor (multiple vendors)
- AxyMats Sealing mat for 96-well microplates (VWR, CAT#14-222-024)
- 96-well PCR microplates (VWR, CAT#14-222-326)

1.2 Solution recipes

- 100mM TrisCl pH 7.5 - 10uL of 1M stock for every 90uL of water (stock, stable at room temperature indefinitely)
- 2M KCl - 745mg in 5mL of water (stock, stable at room temperature indefinitely)
- 0.5M MgCl₂ - 508mg in 5mL of water (stock, stable at room temperature indefinitely)
- 10% (v/v) NP-40 - 10uL for every 90uL of water (stock, stable at room temperature indefinitely)
- 10% (w/v) DOC - 500mg in 5mL of water (stock, stable at room temperature indefinitely)
- 0.1M of dithiothreitol (DTT) - 1.5mg for every 100uL of 100mM TrisCl pH 7.5, prepare fresh (need 10uL per sample)
- 0.4M of chloroacetamide (CAA) - 3.7mg for every 100uL of 100mM TrisCl pH 7.5, prepare fresh (need 10uL per sample)
- 10X cOmplete protease inhibitor stock - 1 tablet in 1mL of water (store at -80C)
- 4M urea (24mg for every 100uL of 100mM TrisCl pH 7.5, prepare just before use)
- Lysis buffer A (need 200uL per sample, recipe for 1mL)
 - 20mM TrisCl pH 7.5 (200uL of 100mM stock)
 - 150mM KCl (76uL of 2M stock)
 - 5mM MgCl₂ (10uL of 0.5M stock)
 - 0.5% (v/v) NP-40 (50uL of 10% stock)
 - 0.5% (v/v) DOC (50uL of 10% stock)
 - 0.5X cOmplete protease inhibitor (50uL of 10X stock)
 - water to 1mL (565uL of clean water)
- Nuclease mix (need 10uL per sample, recipe for 100uL)
 - 100U benzonase (4uL of stock)
 - 50ug RNase A (5uL of stock) (RNase A is optional)
 - water to 100uL
- Lysis buffer B (need 50uL per sample, recipe for 1mL)
 - 600mM TrisCl pH 7.5 (600uL of 100mM stock)
 - 8% (v/v) SDS (400uL of 20% stock)
 - 10mM DTT (6mg of powder)
- 80% (v/v) ethanol (prepare fresh)
- Buffer A - methanol

- Buffer B - 0.1% trifluoroacetic acid (TFA) in water
- Rinse buffer - 0.1% formic acid, 4% methanol, in water
- Elution buffer - 0.1% formic acid in 60% methanol
- Sample Reconstitution Solution - 1% formic acid in water

1.3 Protocol

This protocol assumes you have your cells growing in conventional 6-well dishes. If you are using a 10cm or bigger dish, I suggest harvesting your cells using trypsin and freezing the pellets and proceeding with the processing as below (skipping the cell scraping steps). No matter what vessel you are using, I suggest plating your cells 24h before your planned harvest time to achieve a confluence of 70-80% at harvest time. This will ensure a maximum number of cells are actively growing and producing protein. If your cells are slow to attach or are typically slow to start growing, you can do this 48h before. The end goal is to have an actively growing cell population that is not over-confluent.

If you want a more concentrated cell lysate, use 75uL of Lysis Buffer A in the first step and 25uL of Lysis buffer B later on. Scale as is appropriate based on your cell culture dish size or harvested pellet size.

Prior to starting, prepare sufficient Lysis buffer A and B for all of the samples to be processed. Make sure you have a Thermoblock heated to +95C or greater. Weigh out the CAA and Urea powders into tubes, but don't reconstitute until just before you will use them. Prepare 1 tube for each sample to be processed (protein - 2mL tube).

1. Using a pipette, remove the culture medium from the growing cells on the 6-well plate.
2. Add 2mL of DPBS to each well and swirl the plate to rinse. Pipette up and discard the DPBS (or aspirate).
3. Add 150uL of Lysis buffer A to each well and rock the plate back and forth to ensure the cells are covered.
4. Working one well at a time, use a cell scraper to dislodge the cells and a micropipette to transfer 150uL of lysate to the protein tube on ice. Repeat for each sample.
5. To the 150uL of lysate in the protein tube, add 10uL of nuclease mix and pipette mix. Incubate for 10-minutes at +24C without shaking. OPTIONAL - you can add the nuclease mix directly to the lysis buffer prior to adding it to the cells on the dish and do this incubation while you are scraping the cells off of the plate, in which case, you would skip this step.
6. After incubation, add 50uL of Lysis buffer B to the protein lysate and pipette mix.
7. Place the protein lysate in the pre-heated Thermoblock at +95C and incubate for 5-minutes.
8. Remove the protein lysate from the Thermoblock and let sit at +24C for 10-minutes. During this incubation, reconstitute your CAA powder in an appropriate amount of 100mM TrisCl pH 7.5. It may be difficult to get this entirely into solution, but just get as much as you can.
9. Quick-spin the tubes at low speed to remove any condensation from the lid (e.g. 250g for 30 seconds). At this point, the protein lysate is compatible with Western blotting. I will usually add 6uL of 6X SDS Loading Buffer to 24uL of my sample, and run 20-30uL on a gel.
10. Add 10uL of CAA to the protein lysate and vortex mix. Incubate at +24C for 30-minutes in the dark.
11. Reconstitute your DTT in an appropriate amount of 100mM TrisCl pH 7.5 and add 10uL to each tube of protein lysate and vortex mix. This is a good stop point. The samples here can be stored at -80C indefinitely, or directly used for other protocols such as Western blotting, as mentioned above.
12. To perform clean-up prior to digestion, prepare the SP3 beads stock (if you don't have an existing stock, otherwise skip to Step 14):

1. Vortex the two 15mL containers of bead stocks from the manufacturer to re-suspend the material.
2. Take 500uL of each of the bead stocks and combine in a fresh 2mL tube.
3. Place on a magnetic rack and wait for beads to settle. Discard the supernatant.
4. Reconstitute the beads in 1mL of water with pipetting and place back on the magnetic rack. After the beads settle, discard the supernatant.
5. Repeat the above rinse one additional time for a total of 2 rinses.
6. Reconstitute the beads in 500uL of water. This is your bead stock and it can be stored at +4C indefinitely.
13. To a fresh 2mL tube for each sample, add 10uL of SP3 bead stock and 90uL of your prepared lysate and mix by gentle shaking. The remaining lysate can be stored at -80C for validation assays (e.g. Western blotting), or as a backup sample if your protein digestion fails.
14. Add 400uL of acetone to each tube of protein lysate and gently shake the tube to mix. Incubate at +24C for 5-minutes.
15. During the above incubation, prepare your digestion mixture:
 1. Reconstitute urea powder with an appropriate amount of 100mM TrisCl pH 7.5. Vortex mix. NOTE - urea will increase the volume of the solution generally by the amount of powder you have weighed out. For example, to 240mg of urea, I would add 760uL of 100mM TrisCl pH 7.5 to make 1mL of solution.
 2. Reconstitute a 20ug trypsin/rLysC vial using 100uL of the provided reconstitution solution.
 3. Transfer 5uL of trypsin per sample to a fresh tube, and add 100uL of prepared urea per sample. Vortex mix and keep on ice.
16. Centrifuge the protein lysate tubes at 5,000g for 5-minutes at room temperature.
17. Discard the supernatant from each tube and add 800uL of 80% ethanol. Pipette mix to reconstitute the beads.
18. Centrifuge the tubes at 5,000g for 5-minutes and discard the supernatant. Using a P200 or P20 micropipette, make sure you have removed as much leftover liquid from the rinse as possible from the tubes.
19. Add 100uL of the prepared trypsin/urea mix to each tube. Do not attempt to mix, vortex, or pipette.
20. Transfer the tubes to a shaking Thermomixer set at +30C and 1,000rpm, and digest for 2-4 hours.
21. Add 300uL of 100mM TrisCl pH 7.5 to each tube and place back in the Thermomixer and incubate overnight at +30C and 1,000rpm mixing.
22. The next day, spin the tubes at 12,000g for 2-minutes and then place on a magnetic rack.
23. Clean up your sample using a Strata-X SPE plate:
 1. Add 200uL of Buffer A to the SPE plate wells and centrifuge at 250g for 3-minutes. Empty the collection plate to the waste.
 2. Add 200uL of Buffer B to the SPE plate wells and centrifuge at 250g for 3-minutes. Empty the collection plate to the waste.
 3. Add 200uL of the peptide samples to the SPE plate wells and centrifuge at 250g for 3-minutes. Empty the collection plate to the waste.
 4. Repeat Step 3 to load the remainder of the sample.
 5. Add 200uL of Rinse buffer to the SPE plate wells and centrifuge at 250g for 3-minutes. Empty the collection plate to the waste.
 6. Add 200uL of Elution buffer to the SPE plate wells and centrifuge at 250g for 3-minutes.
 7. Transfer the elution from the collection plate to fresh 1.5mL tubes for each sample.

24. Concentrate the peptide sample by evaporation. You can use a SpeedVac or a Lyophilizer for this purpose.
25. After the sample is evaporated, add 20uL of Sample Reconstitution Solution to the tube, vortex, and then spin at 12,000g for 2-minutes.
26. Transfer 10uL of the reconstituted peptides to a 96-well plate with a silicone mat lid. Freeze the remainder of the peptide material at -80C.
27. Samples for MS analysis in the 96-well plate can be stored at this stage at -20C until analysis.