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Tissue Homogenization and GELFrEE for Top-Down Proteomics

GELFrEE Protocol

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Human BioMolecular Atlas Program (HuBMAP) Method Development Community

Kelleher Research Group

Kelleher KRG Research Group
Northwestern University, National Resource for Translational...

Solid human tissue is homogenized, protein is extracted, and samples prefractionated and desalted. Protein samples are ready for top-down proteomics.

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In steps of

Overall protocol for top-down LC-MS/MS of human lung tissue
Overall protocol for top-down LC-MS/MS of human heart tissue
Overall protocol for top-down LC-MS/MS of human heart tissue
Overall protocol for top-down CZE-MS/MS of human small intestine tissue
Overall protocol for top-down CZE-MS/MS of human spleen tissue

Text written in italic involves critical steps, important observations, relevant information and safety notes.

RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulfate (w/v), pH 7.4, 1X Halt Protease and Phosphotase Inhibitor Cocktail (Thermo Scientific))

HALT phosphatase and protease inhibitor cocktail (100x) **Thermo Fisher Scientific Catalog #78442**

10% GELFrEE cartridge
Acetone (stored in -20C freezer)
Sodium dodecyl sulfate
LCMS -grade methanol (Fisher Scientific #A456-4)
LCMS-grade water (Fisher Scientific #W64)
LCMS-grade acetonitrile (Fisher Scientific #A955-4)
HPLC-grade chloroform
LCMS-grade formic acid (Thermo Scientific #28905)
LCMS-grade acetic acid

Cell lysis, protein precipitation, and lysate quantitation 3h

1 Cryopulverize tissue sample with Retsch Cryomill 3h

Cryomill
Cryogrinder
Retsch **20.749.0001**

1.1 Fill liquid nitrogen dewar and equilibrate to 1.3 bar

1.2 Add frozen tissue sample to 25 mL grinding jar containing a 1-inch stainless steel ball.

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1.3 Pulverize with three cycles of precooling with liquid nitrogen at 1 Hz for 3 min and grinding at 30 Hz for 1 min

2 Transfer pulverized tissue to a 15 mL conical tube and resuspend in 2 mL of cold RIPA cell lysis buffer (including 1x HALT protease/phosphatase inhibitor cocktail).

3 Sonicate lysate with a probe sonicator on ice (40% power, cycle 2 sec on, 3 sec off, 30 sec total).

Fisherbrand Model 120 Sonic Dismembrator
Sonicator
Fisherbrand **FB120110**
Equipped with 1/8" probe

4 Transfer lysate to 1.5 mL Lo-bind Eppendorf tube.
Protein LoBind Tubes, 1.5 mL **Eppendorf Catalog #0030108116**

5 Transfer two 200 uL aliquots from each sample lysate to fresh 1.5mL Lo-bind Eppendorf tubes

6 Add 6 volumes of ice-cold acetone to the lysates, vortex at high speed (setting 8) for 10–20 s, and place the tubes in a −80 °C freezer for at least 30 min to precipitate the proteins. *The protein precipitate is stable in acetone overnight*.

Analog Vortex Mixer
Vortex mixer
Fisherbrand **02-215-365**

7 Remove the tubes from the freezer and immediately centrifuge **21000 x g, 4°C, 00:30:00** 30m

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8 Remove as much acetone as possible from the protein pellets without disturbing them. Place tubes in a biological safety cabinet for 5–10 min to evaporate off any excess acetone.

9 Resuspend the protein pellet in 500 µL of 1% (wt/vol) SDS solution with pipetting and probe sonication *It may take multiple rounds of vigorous pipetting to completely dissolve the precipitates. Lysates my also be stored at -80C until further processing.*

10 Transfer 25 µL of the solution to a new 1.5-mL Protein LoBind tube for the BCA assay. The tubes containing the remaining 200 µL of resuspended proteins should be kept on ice.

11 A BCA assay should now be performed on the 25-µL aliquots of sample proteome, using a 96-well plate and following the manufacturer's recommended protocol.

12 After collection of colorimetric data for BCA assay on a 96-well-plate reader, create a standard curve, and fit the average of the triplicate values for each sample dilution to the standard curve to determine the protein concentration of each sample, after correcting for dilutions.

Protein fractionation by GELFrEE 3h

13 Calculate 300 ug protein

14 Transfer the calculated volume containing 300 µg of protein to a new 1.5-mL Protein LoBind tube. *The leftover lysates in 1% (wt/vol) SDS can be stored for several months at −80 °C. From this point on, keep the sample tubes on ice until immediately before GELFrEE.*

15 Add 8 µL of 1 M DTT, 30 µL of GELFrEE sample loading buffer, and dH2O to a final volume of 150 µL.

16 Vortex each tube for 10–20 s, and heat it at 95 °C for 5 min. Centrifuge the samples at 1,000g for 2 min at 4 °C to re-collect any condensation at the tube lids after heating

17 Perform fractionation by GELFrEE by following the manufacturer's protocol for 10% cartridges.

GELFrEE 8100 Fractionation System
Fractionator
Protein Discovery **42001**

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18 After fractions 1-5 of each sample has been collected into a new, 1.5-mL Protein LoBind tube, the fracationation process can end or more fractions can be collected, depending on the experiment. *This is a natural stopping point in the protocol, as GELFrEE fractions are stable for several months at −80 °C.*

Quality control of GELFrEE fraction by SDS–PAGE with silver staining 2h

19 Mix 5–10 µL of each fraction of interest with the appropriate amount of Laemmli sample buffer for the gel lane capacity in a new microcentrifuge tube. Boil these gel samples at 95 °C for 5 min and spin down (5,000g, 20–25 °C, 30–60 s).

20 Separate the proteins in each fraction by MW using SDS–PAGE, being careful not to run lower-MW proteins off the gel that may be of interest for TDMS or TDP characterization.

21 Remove the gel and submerge it in fixing solution in a clean container. Place on an orbital shaker for 20 min at low speed to fix the gel

22 Replace the fixing solution with enough washing solution to submerge the gel. Shake for 10 min at RT.

23 Replace the washing solution with sensitizing solution. Shake for 1 min at low speed to sensitize the gel.

24 Pour out the sensitizing solution and rinse the gel with two washes of dH2O for 1 min each.

25 Add enough silver nitrate solution to submerge the gel and incubate it for 20 min on an orbital shaker at low speed to stain the gel.

26 Pour out the silver nitrate solution and rinse the gel twice in dH2O for 1 min.

27 Add enough developing solution to submerge the gel, and develop under rapid shaking. *Discard the developing solution and replace it with fresh developing solution, once the liquid turns yellow, to reduce background staining.*

28 Pour out the developing solution and replace with terminating solution, once the desired staining intensity has been achieved, to terminate the development . The gel can now be scanned or photographed.

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Desalting and concentrating GELFrEE fractions 2h

29 To each fraction, add 4 volumes of Optima-grade methanol (600 µL), and vortex each tube vigorously for 20 s.

30 Add 1 volume (150 µL) of chloroform to each fraction, and vortex vigorously for 20 s.

31 Add 3 volumes (450 µL) of LC–MS-grade water to each fraction. The liquid should immediately become cloudy and white in color. Vortex vigorously for 20 s

32 Centrifuge the tubes at 21,000g for 10 min at 4 °C.

33 Carefully remove the tubes from the centrifuge. The separated aqueous and organic phases should be clearly observed, with a visible white protein pellet floating at the interface*. Be very careful when transporting tubes to avoid shaking them or otherwise disturbing the protein pellet more than is necessary*.

34 Remove and discard the top layer of solution, using extreme care to not remove or disturb the protein pellet. It may be necessary to leave a few millimeters of volume above the pellet to avoid accidentally pipetting it out.

35 Very slowly add 3 volumes (450 µL) of Optima methanol to the tube, to avoid breaking up the protein pellet more than is necessary. Slowly mix the solution by carefully rocking the tube back and forth three to five times, while keeping an eye on the protein pellet and being careful to break it up as little as possible.

36 Centrifuge the tubes at 21,000g for 10 min at 4 °C

37 At this point, the protein pellet should now be at the bottom of the tube. Carefully remove and discard as much methanol as possible from the tube without disturbing the pellet, and repeat steps 32–34 two more times.

38 After the final methanol wash, remove as much methanol as possible from the tube without accidentally removing protein pellet particles. Place open tubes in a tube rack in an operating biological safety cabinet for 5–10 min to evaporate off any excess methanol.

39 If sample is to be analyzed by CZE-MS/MS, pellet is resuspended in 10 uL 0.3% acetic acid. If sample is to be analyzed by LC-MS/MS, pellet is resuspended in 25 uL of buffer A (5% acetonitrile, 94.8% water,

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0.2% formic acid).

40 Centrifuge the samples at 21,000g for 5 min at 4 °C, and slowly remove the majority of the solution, pipetting from just below the surface to avoid disturbing any unnoticed particles. Transfer each sample to a separate, pre-chilled LC vial.

41 Transport the vials on ice to the nano-UHPLC auto sampler and analyze immediately to prevent sample degradation.

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