Sep 01, 2022

Top Down Proteomics Data Collection for Microdissected Pancreas Tissue Functional Units

Forked from Top Down Proteomics Data Collection for Microdissected Kidney Tissue Functional Units

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https://dx.doi.org/10.17504/protocols.io.261ge3pw7l47/v1

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Human BioMolecular Atl... PNNL-TTD

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

The protocol describes how to use laser capture microdissection (LCM) to cut small regions of interest (~200-300 µm) from tissue sections. This is followed by top down proteomics analysis by liquid chromatography - mass spectrometry (LC-MS).

Materials

- LC solvents

Mobile phase A (MPA): 0.2% formic acid in water (LCMS grade)
Mobile phase B (MPB): 0.2% formic acid in acetonitrile (LCMS grade)

- Instrumentation

Equipment	
NanoAcquity	NAME
liquid chromatography	TYPE
Waters	BRAND
186016002	SKU
Dual pump configuration with autosampler 186016007	SPECIFICATIONS

Equipment	
Orbitrap Lumos	NAME
Mass spectrometer	TYPE
Thermo	BRAND
IQLAAEGAAPFADBMBHQ	SKU
https://www.thermofisher.com/order/catalog/product/IQLAAEGAAPFADBMBHQ	LINK

- QC sample

Shewanella oneidensis MR-1 cell culture
Homogenization buffer (HB) : 8M urea solution (480 mg/mL) in 50 mM ammonium bicarbonate with 15 mM TCEP
Wash Buffer (WB): 0.2% formic acid, 5% acetonitrile (LC-MS grade solvents)

Liquid chromatography (LC) method setup

1 Set up reversed-phase LC system with online trapping for desalting.

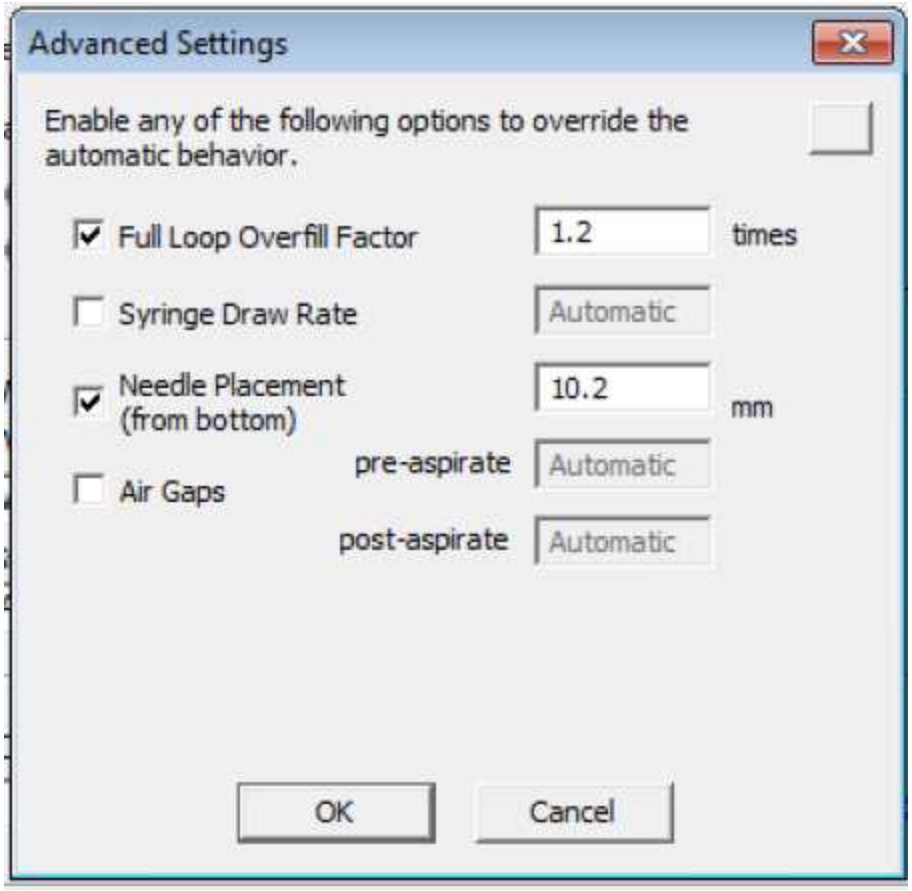
Dual pump configuration
Mobile phase A (MPA): 0.2% formic acid in water (LCMS grade)
Mobile phase B (MPB): 0.2% formic acid in acetonitrile (LCMS grade)

Equipment	
NanoAcquity	NAME
liquid chromatography	TYPE
Waters	BRAND
186016002	SKU
Dual pump configuration with autosampler 186016007	SPECIFICATIONS

1.1 Prepare the method for autosampler for microPOTS samples.

Note

For samples processed by the microPOTS protocol cited below, the LC vials will hold PCR tubes inside. The height of the syringe in the autosampler must be adjusted to avoid damage to the needle. This can be accessed within the nanoACQUITY Sample Manager Software. Select the autosampler and the "advanced" options. Under this tab, check the "Needle Placement (from bottom)" box. Adjust the needle placement to 10.2 mm from the bottom of the LC vial.



Protocol

NAME

Laser Capture Microdissection of Tissue Functional Units for microPOTS Top-Down Proteomics

CREATED BY

james.fulcher

Preview

1.2 Set up gradient method for samples. 10m

Wash pump: 5 µL/min, 95% MPA, 5% MPB. Loading time 10 min.

Gradient pump: 0.3 µL/min
0 min: 95% MPA, 5% MPB
1 min: 90% MPA, 10% MPB
90 min: 40% MPA, 60% MPB
100 min: 95% MPA, 5% MPB

Note

Blank runs with high % MPB into both the trap and the analytical column can be added in between samples to minimize carry over. However, for the low sample loading in this case, blank is not absolutely necessary.

Mass spectrometer (MS) method setup

2 Calibrate and set up the mass spectrometer method for sample runs.

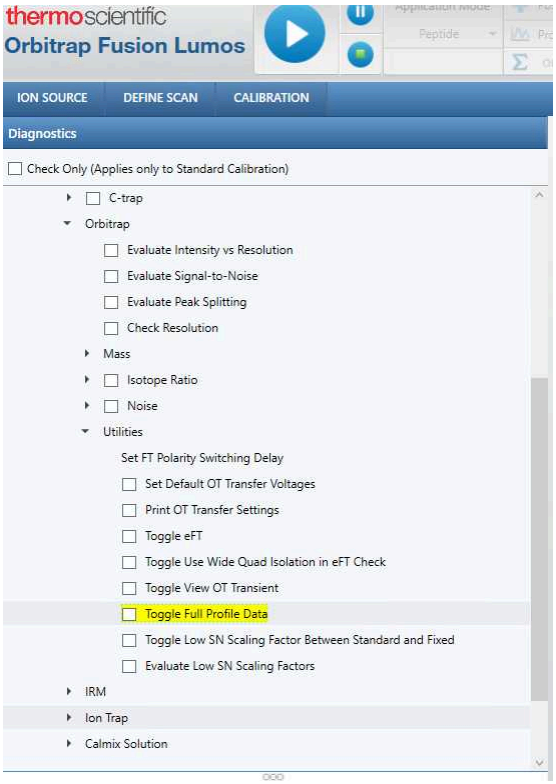
2.1 Perform both mass and system calibration following instrument vendor's recommendation. 30m

Note

At minimum, "positive polarity" and "Orbitrap mass" calibrations need to be completed. System calibration is strongly recommended to ensure good performance for "Intact protein mode" and ETD.

2.2 (Optional) Turn on full profile mode. 1m

Under "Diagnosis" - "System" - "Orbitrap" - "Utilities", check "Toggle Full Profile Data" before starting the queue.



Note

Full profile mode will generate ~10 GB files per 100 min run. The raw data will save all the baseline signal (including noise), which may increase the likelihood of capturing low abundance species. Remember to toggle off the full profile mode after the queue to reset the instrument for regular experiments.

2.3 Set up the data dependent acquisition method with the following parameters.

ETD method was used to obtain higher sequence coverage than CID for characterizing proteoforms. In addition, a ETD method with inclusion list for histones was used on some replicates to improve the analysis of histone modifications.

Sample method - CID

Note

Global Settings
Use Static Source Gasses
Use Ion Source Settings from Tune = Checked
Method Duration (min)= 100
Spray Voltage = Static
Gas Mode = Static
Infusion Mode (LC)= False
FAIMS Mode = Not Installed
Application Mode = Peptide
Pressure Mode = Standard
Default Charge State = 6
Advanced Peak Determination = True
Experiment 1
Experiment Name = MS
Start Time (min) = 0
End Time (min) = 100

Scan MasterScan
Desired minimum points across the peak = 6
MSn Level = 1
Use Wide Quad Isolation = True
Detector Type = Orbitrap
Orbitrap Resolution = 120K
Mass Range = Normal
Scan Range (m/z) = 500-2000
Maximum Injection Time (ms) = 500
AGC Target = 1000000
Normalized AGC Target = 250%
Microscans = 2
Maximum Injection Time Type = Custom
RF Lens (%) = 30
Use ETD Internal Calibration = False
DataType = Profile
Polarity = Positive
Source Fragmentation = False
Scan Description =
Enhanced Resolution Mode = Off

Filter ChargeState
Include charge state(s) = 3-35
Include undetermined charge states = False

Filter DynamicExclusion
Exclude after n times = 1
Exclusion duration (s) = 30
Mass Tolerance = mz
Mass tolerance low = 1
Mass tolerance high = 1
Use Common Settings = False
Exclude isotopes = True
Perform dependent scan on single charge state per precursor only = True

Data Dependent Properties
Data Dependent Mode= Number of Scans
Number of Dependent Scans= **1 (for sample run); 4 or 8 (for library run)**
Scan Event 1

Scan ddMSnScan
Desired minimum points across the peak = 6
MSn Level = 2
Isolation Mode = Quadrupole
Enable Intelligent Product Acquisition for MS2 Isolation = False
Isolation Window = 2
Isolation Offset = Off
Reported Mass = Original Mass
Multi-notch Isolation = False
Scan Range Mode = Auto
Scan Priority= 1
Collision Energy Mode = Fixed
ActivationType = CID
Collision Energy (%) = 35
Activation Time (ms) = 10
Activation Q = 0.25
Multistage Activation = False
Detector Type = Orbitrap
Orbitrap Resolution = 60K
Maximum Injection Time (ms) = 200
AGC Target = 500000
Inject ions for all available parallelizable time = False
Normalized AGC Target = 1000%
Microscans = 1
Maximum Injection Time Type = Custom
Use ETD Internal Calibration = False
DataType = Profile
Polarity = Positive
Source Fragmentation = False
Scan Description =
Time Mode = Unscheduled
Enhanced Resolution Mode = Off

Sample method - ETD

Note

Global Settings
Use Static Source Gasses
Use Ion Source Settings from Tune = Checked
Method Duration (min)= 100
Spray Voltage = Static
Gas Mode = Static
Infusion Mode (LC)= False
FAIMS Mode = Not Installed
Application Mode = Peptide
Pressure Mode = Standard
Default Charge State = 6
Advanced Peak Determination = True
Experiment 1
Experiment Name = MS
Start Time (min) = 0
End Time (min) = 100

Scan MasterScan
Desired minimum points across the peak = 6
MSn Level = 1
Use Wide Quad Isolation = True
Detector Type = Orbitrap
Orbitrap Resolution = 120K
Mass Range = Normal
Scan Range (m/z) = 500-2000
Maximum Injection Time (ms) = 200
AGC Target = 1000000
Normalized AGC Target = 250%
Microscans = 2
Maximum Injection Time Type = Custom
RF Lens (%) = 30
Use ETD Internal Calibration = False
DataType = Profile
Polarity = Positive
Source Fragmentation = False
Scan Description =
Enhanced Resolution Mode = Off

Filter ChargeState
Include charge state(s) = 5-35
Include undetermined charge states = False

Filter IntensityThreshold
Maximum Intensity = 1E+20
Minimum Intensity = 20000
Relative Intensity Threshold = 20
Intensity Filter Type = IntensityThreshold

Filter DynamicExclusion
Exclude after n times = 1
Exclusion duration (s) = 30
Mass Tolerance = mz
Mass tolerance low = 1

Mass tolerance high = 1
Use Common Settings = False
Exclude isotopes = True
Perform dependent scan on single charge state per precursor only = True

Data Dependent Properties
Data Dependent Mode= Number of Scans
Number of Dependent Scans= 5
Scan Event 1

Filter PrecursorPriority
HighestChargeState

Scan ddMSnScan
Desired minimum points across the peak = 6
MSn Level = 2
Isolation Mode = Quadrupole
Enable Intelligent Product Acquisition for MS2 Isolation = False
Isolation Window = 2
Isolation Offset = Off
Reported Mass = Original Mass
Multi-notch Isolation = False
Scan Range Mode = Auto
Scan Priority= 1
ActivationType = ETD
Use calibrated charge dependent ETD parameters = False
ETD Reaction Time (ms) = 15
ETD Reagent Target = 700000
Max ETD Reagent Injection Time (ms) = 200
ETD Supplemental Activation = False
Detector Type = Orbitrap
Orbitrap Resolution = 60K
Maximum Injection Time (ms) = 250
AGC Target = 500000
Inject ions for all available parallelizable time = False
Normalized AGC Target = 1000%
Microscans = 1
Maximum Injection Time Type = Custom
Use ETD Internal Calibration = False
DataType = Profile
Polarity = Positive
Source Fragmentation = False
Scan Description =
Time Mode = Unscheduled
Enhanced Resolution Mode = Off

Histone target inclusion list (H4 and H3) for ETD method

Note

>>>>>>>>>>>>> Mass List Table <<<<<<<<<<<<<

CompoundName	m/z	t start (min)	t stop (min)
H4 16+	706.72	41	45
H4 Ac me2	707.59	41	45
H4 Ac me3	708.47	41	45
H4 Ac me4	709.34	41	45
H4 Ac me5	710.22	41	45
H4 Ac me6	711.1	41	45
H4 Ac me7	712.03	41	45
H4 Ac me8	712.91	41	45
H4 17+	665.21	41	45
H4 Ac me3	666.85	41	45
H4 Ac me4	667.68	41	45
H4 Ac me5	668.5	41	45
H4 Ac me6	669.33	41	45
H4 Ac me7	670.2	41	45
H4 Ac me8	670.97	41	45
H3 me1 23+	662.2	50	55
H3 me2	662.9	50	55
H3 me3	663.51	50	55
H3 me4	664.12	50	55
H3 me5	664.77	50	55
H3 me6	665.38	50	55
H3 me7	665.99	50	55
H3 me8	666.6	50	55
H3 me9	667.21	50	55
H3 me10	667.86	50	55
H3 me11	668.47	50	55
H3 me12	669.08	50	55
H3 me13	669.68	50	55
H3 me14	670.38	50	55
H3 me15	670.9	50	55
H3 me16	671.55	50	55
H3 me1 22+	692.39	50	55
H3 me2	692.94	50	55
H3 me3	693.67	50	55
H3 me4	694.26	50	55
H3 me5	694.94	50	55
H3 me6	695.58	50	55
H3 me7	696.21	50	55
H3 me8	696.85	50	55
H3 me9	697.49	50	55
H3 me10	698.17	50	55
H3 me11	698.76	50	55
H3 me12	699.44	50	55
H3 me13	700.17	50	55
H3 me14	700.81	50	55
H3 me15	701.4	50	55
H3 me16	701.94	50	55

>>>>>>>>>>>>> End Mass List Table <<<<<<<<<<<<<

Instrument Quality Control (QC) and method setup

3 A QC standard is used to evaluate instrument performance before starting samples. Herein we use a bacterial lysate established in our lab (see reference below for more information), other samples can be used as QC as well.

Citation

Shen Y, Tolić N, Piehowski PD, Shukla AK, Kim S, Zhao R, Qu Y, Robinson E, Smith RD, Paša-Tolić L
(2017)
. High-resolution ultrahigh-pressure long column reversed-phase liquid chromatography for top-down proteomics.
Journal of chromatography. A.

https://doi.org/10.1016/j.chroma.2017.01.008 LINK

QC Sample information

Intact protein lysate from cultured Shewanella oneidensis MR-1 cells

Buffer preparation:
Homogenization buffer (**HB**) : 8M urea solution (480 mg/mL) in 50 mM ABC with 15 mM TCEP
Note: Use BondBreaker 0.5 M TCEP stock solution

Wash Buffer (**WB**): 0.2% formic acid, 5% acetonitrile
Note: Use LC-MS grade water

Note

NOTE: Adjust centrifugal filtration speeds and times as appropriate for your sample type and filter size.It is recommended to do all spin steps at 10 °C (8 M urea will freeze at 4 °C).

1. Lyse cells or homogenize tissue in homogenization buffer (HB).
2. Incubate sample at room temperature for 30 min to extract and denature proteins
3. Centrifuge lysate at 14,000 x G, 10C for 10 minutes to pellet cell debris
4. Transfer supernatant to 100K MWCO filter and centrifuge at 14,000 x G until minimum volume is reached.
5. Wash 100K spin filter with 1X max volume of HB, spin at 14,000 x G until minimum volume is reached.
6. Transfer filtrate from 100K filter to a fresh 10K filter and centrifuge at 14,000 x G for time needed to get to minimum volume.
    a. If needed, add multiple aliquots of filtrate from 100K filter to the same 10K filter
7. Wash 10K filter three times with wash buffer (WB) and spin to minimum volume each wash.
8. Perform Coomassie or BCA protein assay.
9. Dilute sample to 0.01 ug/uL in WB and aliquot 100 uL into separate 0.6 mL Eppendorf tubes with labels.

3.1 QC LCMS method

LC method

Wash pump: 5 µL/min, 95% MPA, 5% MPB. Loading time 15 min.

Gradient pump: 0.3 µL/min
0 min: 95% MPA, 5% MPB
180 min: 55% MPA, 45% MPB

Followed by a blank injection to wash the column before sample runs

MS method

Note

Global Settings
Use Static Source Gasses
Use Ion Source Settings from Tune = Checked
Method Duration (min)= 180
Spray Voltage = Static
Gas Mode = Static
Infusion Mode (LC)= False
FAIMS Mode = Not Installed
Application Mode = Intact Protein
Pressure Mode = Low Pressure
Default Charge State = 10
Advanced Peak Determination = True
Experiment 1
Experiment Name = MS
Start Time (min) = 0
End Time (min) = 180

Scan MasterScan
Desired minimum points across the peak = 6
MSn Level = 1
Use Wide Quad Isolation = True
Detector Type = Orbitrap
Orbitrap Resolution = 120K
Mass Range = Normal
Scan Range (m/z) = 500-2000
Maximum Injection Time (ms) = 400
AGC Target = 1000000
Normalized AGC Target = 250%
Microscans = 4
Maximum Injection Time Type = Custom
RF Lens (%) = 30
Use ETD Internal Calibration = False
DataType = Profile
Polarity = Positive
Source Fragmentation = True
Energy (V) = 15
Scan Description =
Enhanced Resolution Mode = Off

Filter ChargeState
Include charge state(s) = 4-35
Include undetermined charge states = False

Filter DynamicExclusion
Exclude after n times = 1
Exclusion duration (s) = 30
Mass Tolerance = mz
Mass tolerance low = 1.5
Mass tolerance high = 1.5
Use Common Settings = False
Exclude isotopes = True

Perform dependent scan on single charge state per precursor only = True

Data Dependent Properties
Data Dependent Mode= Number of Scans
Number of Dependent Scans= 4
Scan Event 1

Scan ddMSnScan
Desired minimum points across the peak = 6
MSn Level = 2
Isolation Mode = Quadrupole
Enable Intelligent Product Acquisition for MS2 Isolation = False
Isolation Window = 3
Isolation Offset = Off
Reported Mass = Original Mass
Multi-notch Isolation = False
Scan Range Mode = Define m/z range
Scan Priority= 1
Collision Energy Mode = Fixed
ActivationType = CID
Collision Energy (%) = 35
Activation Time (ms) = 10
Activation Q = 0.25
Multistage Activation = False
Detector Type = Orbitrap
Orbitrap Resolution = 60K
Scan Range (m/z) = 400-2000
Maximum Injection Time (ms) = 200
AGC Target = 500000
Inject ions for all available parallelizable time = False
Normalized AGC Target = 1000%
Microscans = 2
Maximum Injection Time Type = Custom
Use ETD Internal Calibration = False
DataType = Profile
Polarity = Positive
Source Fragmentation = False
Scan Description =
Time Mode = Unscheduled
Enhanced Resolution Mode = Off

Data collection and QC metric

4 Queue the QC sample before starting the samples using the LCMS method described in section 3. QC runs need to pass the metrics defined below. Once passed, queue the sample runs using the LCMS method described in section 2.

4.1 Evaluation of QC data (*Shewanella* lysate from section 3)

To quickly evaluate the QC data, open them in the Thermo Scientific Freestyle software or Xcalibur QualBrowser. The following metrics are inspected to ensure that the LCMS run meets the expected standard.

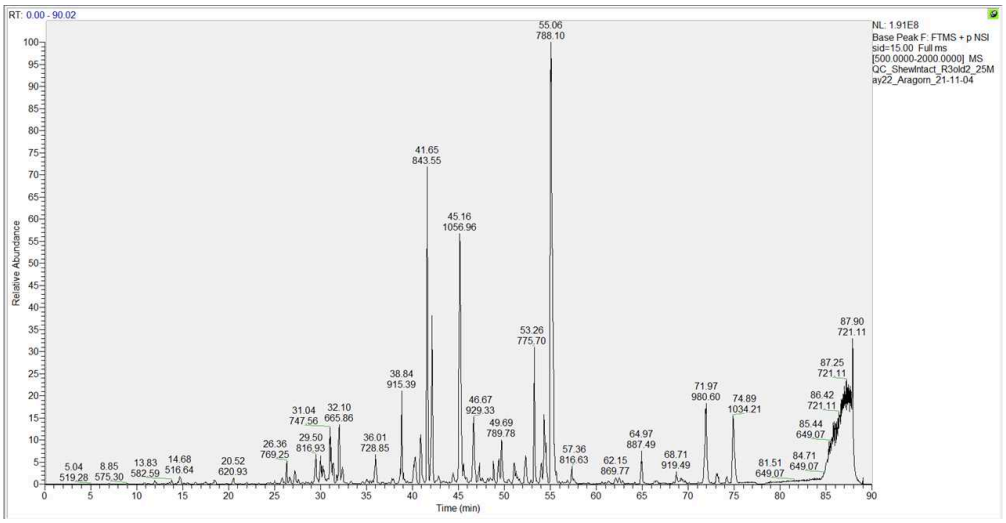
1. Signal levels

The intensity levels at the total ion current (TIC), base peak intensity (BPI), and the MS2 spectra are inspected to ensure that they meet the expected intensity levels. The TIC intensity levels are typically expected to be above the 1e10 level, BPI at or above 1e8 level, and the MS2 spectra ion current from 1e5 to 1e7 levels.

2. Chromatography

2a.

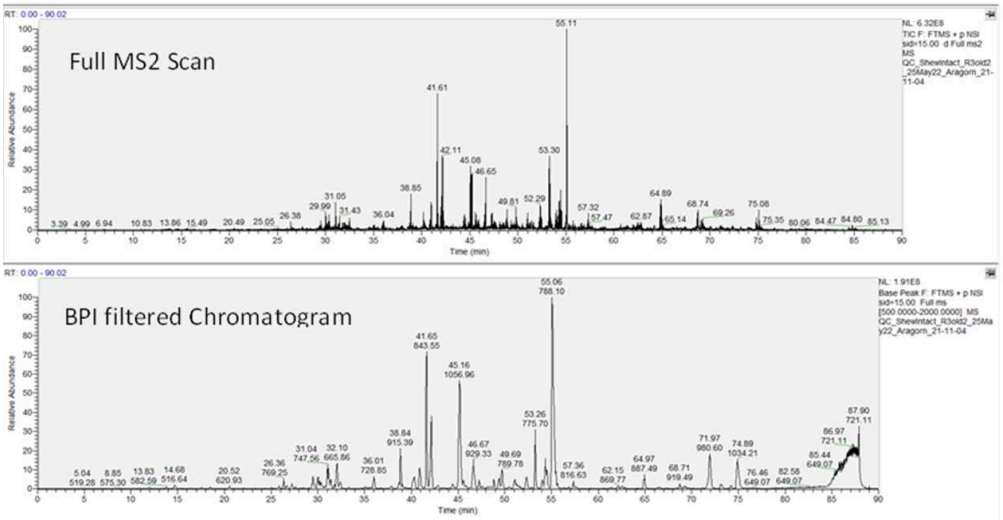
We look at the chromatography distribution using the base peak FTMS scan as filter to ensure that the eluted peaks are well distributed through the LC run. The chromatogram is filtered to show the BPI peaks and inspected to ensure no peak broadening (early or late) is observed.



Chromatogram filtered using the BPI to show the peak distribution.

2b.

The distribution of the MS2 peaks (fragmentation of peaks selected at the MS1 level) is inspected to evaluate how similar the distribution is to that of the MS1 level. The MS2 distribution is expected to emulate what is observed for the peak distribution of the MS1 when the BPI filter is applied, indicating that peak selection for fragmentation was performed at an appreciable level. To assess the MS2 fragmentation signal, the “full MS2” is applied as the filter to show the MS2 distribution through the whole experiment.



2c.
Finally, QC samples are analyzed with TopPIC to ensure appropriate number of proteoforms and proteoform spectrum matches (PrSMs) are being identified.

Software

TopPIC Suite	NAME
Xiaowen Liu	DEVELOPER
https://github.com/toppic-suite/toppic-suite	REPOSITORY
https://www.toppic.org/software/toppic/index.html	SOURCE LINK

Proteoforms are counted by opening the exported "..._proteoforms.tsv" file and PrSMs through the "..._PrSMs.tsv" file. The QC passing threshold for proteoforms is 1,000 and 2,000 for PrSMs.

Quality Assurance (QA) of HubMAP Samples

5 Perform proteoform identification using the "TopPIC processing" section in the following protocol.

Citation

James M Fulcher, Yen-Chen Liao, Mowei Zhou, Ljiljana.PasaTolic (2026)
. Proteoform Identification and Quantitation with TopPIC and TDPortal for Human Tissues.

dx.doi.org/10.17504/protocols.io.3byl4bpj2vo5/v1 LINK

5.1 Using the TopPIC PrSM results (after TopPICR post-processing), kidney samples are filtered based on the total number of PrSMs. A cutoff of 100 PrSMs was used to remove samples that were of lower quality.

Citations

Step 3

Shen Y, Tolić N, Piehowski PD, Shukla AK, Kim S, Zhao R, Qu Y, Robinson E, Smith RD, Paša-Tolić L. High-resolution ultrahigh-pressure long column reversed-phase liquid chromatography for top-down proteomics. **https://doi.org/10.1016/j.chroma.2017.01.008**

Step 5

James M Fulcher, Yen-Chen Liao, Mowei Zhou, Ljiljana.PasaTolic. Proteoform Identification and Quantitation with TopPIC and TDPortal for Human Tissues
dx.doi.org/10.17504/protocols.io.3byl4bpj2vo5/v1