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Proteomics Sample Preparation of Human Skin Punch Biopsies for Data-Independent Acquisition Mass Spectrometry Analysis

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Matthew Jaconelli[1], Francesca Tonelli[1], Dario Alessi[1]

[1]MRC-PPU, University of Dundee

Francesca Tonelli
MRC-PPU at The University of Dundee

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Protocol status: Working
We use this protocol and it's working

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Funders Acknowledgements:
Michael J Fox Foundation for Parkinson's Research

Disclaimer

Abstract

Protocol for the processing of human skin tissue punch biopsies from the C8 paravertebral region for data-independent acquisition mass spectrometry (DIA-MS) proteomics, including generation of a human skin proteome MS spectral library. This protocol was generated as part of a pilot study in collaboration with the Michael J. Fox Foundation. The biospecimens were obtained from the Parkinson's Progression Marker Initiative (PPMI) (RRID:SCR_006431). For up-to-date information on the study, visit **www.ppmi-info.org**.

Materials

Acetonitrile ≥99.9% **VWR International (Avantor)** **Catalog #**1.00030.2500

Formic Acid, 99.0+%, Optima™ LC/MS Grade ThermoFisherScientific Catalog#A117-50

LC-grade Water **Fisher Scientific** **Catalog #**10777404

Ammonium formate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**70221-25G-F

Thermo Scientific™ EPA Screw Vial Assembled Kit, Vials/Septa/Caps **Thermo Scientific** **Catalog #**11543750

Precellys Lysing Kit Catalog#P000922-LYSK0-A

Sodium Dodecyl Sulfate (SDS), Micropellets, Fisher BioReagents **Thermo Fisher Scientific** **Catalog #**15450685

HEPES **Formedium** **Catalog #**HEPES10

Bond-Breaker™ TCEP Solution, Neutral pH **Thermo Fisher Scientific** **Catalog #**77720

Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I1149-25G

Trifluoroacetic acid for HPLC > 99.0% **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**302031-100ML

Methanol, for HPLC-MS, Fisher Chemical™ **Thermo Fisher Scientific** **Catalog #**10653963

Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T7408-500ML

Thermo Scientific™ Mass Spectrometry Grade Proteases **Thermo Fisher Scientific** **Catalog #**15956915

n-Dodecyl-beta-Maltoside Detergent **Thermo Fisher** **Catalog #**89902

S-Trap™ Micro Column (≤ 100 µg) **Protifi** **Catalog #**C02-micro-80

cOmplete™ EDTA-free Protease Inhibitor Cocktail **Roche** **Catalog #**11873580001

Roche PhosSTOP™ **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**4906837001

Eppendorf™ twin.tec™ PCR Plates LoBind™ - PCR Plates **Thermo Fisher Scientific** **Catalog #**15280735

Pierce BCA Protein Assay Kit **Thermo Fisher Scientific** **Catalog #**23225

SafeSeal reaction tube, 2 ml, PP, PCR Performance Tested, Low protein-binding **Sarstedt** **Catalog #**72.695.600

Acclaim™ PepMap™ 100 C18 LC Columns, 3µm, 20mm length, 0.075mm I.D. **Thermo Fisher** **Catalog #**164535

Thermo Scientific™ EASY-Spray™ PepMap™ Neo UHPLC Columns **Thermo Scientific** **Catalog #**17477583

XBridge BEH C18 Column 130Å 3.5 µm 4.6 mm X 250 mm 1/pk **Waters** **Catalog #**186003943

Note

All solvents and buffers were prepared in or aliquoted from glass amber vials, using sterile (not autoclaved) pipette tips or glass cylinders.

Equipment/Software:

Vanquish Neo nano-liquid chromatography system (ThermoScientific)
Orbitrap Astral Mass Spectrometer (ThermoScientific)
Vanquish liquid chromatography system inline with fraction collector module (ThermoScientific)
ThermoMixer C (Eppendorf)
Savant SpeedVac SPD140DDA Vacuum Concentrator (ThermoScientific)
Micro Star 17 microcentrifuge (VWR)
Centurion Scientific Limited centrifuge
Precellys Evolution tissue homogeniser with Cryolys Evolution (Bertin Technologies)
BioRuptor Plus with minichiller 300 (Diagenode)
Xcalibur (ThermoScientific)
DIA-NN (version 1.8.1)

Protein Extraction

27m

1 Transfer frozen skin tissue to a Precellys Lysing Kit (Bertin Technologies) 2ml ceramic bead tube containing 500 µL SDS lysis buffer: 2% SDS (w/v), 20 millimolar (mM) HEPES pH 8, with complete EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor cocktail tablets (Roche).

> Note
>
> Place samples On ice immediately following transfer.

2 Immediately transfer samples to the Precellys Evolution tissue homogeniser (Bertin Technologies). Lyse tissue at 0 °C for 15× 30 second cycles at 6800 rpm, with 30 second rest between each cycle.

3 Centrifuge samples at 2000 x g, 4°C, 00:02:00 to reduce bubbles and transfer supernatant to fresh Protein LoBind Eppendorf tubes (Sarstedt).

2m

4 Heat samples at 95 °C for 00:05:00 on a heat-block (Grant, QBT2).

5m

5 Sonicate samples using a BioRuptor Diagenode (Diagenode) at 4 °C for 15× 30 second cycles, with 30 seconds rest between each cycle.

6 Centrifuge samples at 17000 x g, 4°C, 00:20:00 and carefully transfer supernatant to fresh Protein LoBind Eppendorf tubes (Sarstedt), taking care not to disturb the pelleted cell debris or any visible lipids.

20m

7 Quantify protein concentrations using BCA assay (ThermoScientific).

> Note
>
> Samples can be stored at -80 °C at this stage.

8 Aliquot 20 µg protein from each sample for total proteomic analysis.

Total Proteomics Sample Preparation

1h

9 Adjust total volume to 40 µL using SDS lysis buffer.

10 Reduce samples with [M] 10 millimolar (mM) TCEP (final concentration, [M] 100 millimolar (mM) TCEP stock in [M] 300 millimolar (mM) TEABC) for 1100 rpm, 60°C, 00:30:00 (ThermoMixer C, Eppendorf).

30m

11 Alkylate samples in the dark with [M] 40 millimolar (mM) iodoacetamide (final concentration, 400mM stock) for 1100 rpm, 25°C, 00:30:00 (ThermoMixer C, Eppendorf).

30m

12 Add 20% SDS to achieve a final concentration of 5% SDS, then acidify samples through addition of Trifluoracetic acid to a final concentration of 1%.

Micro S-Trap On-Column Digest

1h 22m

13 Add 6x sample volume of wash buffer (90% methanol, 10% 100mM TEABC).

14 Mix sample by pipetting up and down, then load 150 µL of sample onto micro-columns inside fresh 2ml Protein LoBind Eppendorf tubes (Sarstedt) within a centrifuge.

15 Centrifuge at 1000 x g, 00:01:00 and discard flow-through.

1m

16 Repeat go to step #15 until all sample has been loaded through micro-column.

17 Wash S-Trap columns with bound protein by adding 150 µL wash buffer followed by centrifugation at 1000 x g, 00:01:00 . Discard flow-through. 1m

18 Repeat step 17 three times.

Note

Ensure there is no remaining wash buffer in the S-trap micro columns before proceeding to step 19, as this may impact digestion efficiency.

19 Digest protein by addition of 60 µL (1.5µg) of Trypsin/Lys-C mix (MS grade, Promega, UK) in [M] 50 millimolar (mM) TEABC solution (pH 8) at 47 °C for 01:20:00 , followed by incubation at 22 °C Overnight (approximately 16 hours). 1h 20m

Peptide Elution 4m

20 Elute samples by addition of 40 µL [M] 50 millimolar (mM) TEABC (pH 8) and centrifuge at 1000 x g, 00:01:00 . 1m

Note

Do NOT discard flow-through containing peptides at any step of elution.

21 Add 40 µL 0.15% (v/v) formic acid (FA) and centrifuge at 1000 x g, 00:01:00 . 1m

22 Add 40 µL 80% acetonitrile (ACN), 0.15% FA and centrifuge at 1000 x g, 00:01:00 . Repeat twice (3x total). 1m

23 Dry peptides at Room temperature using a vacuum centrifuge and store dried peptides at -20 °C until mass spectrometry analysis.

Peptide Resuspension

30m

24 Resuspend samples by addition of 100 µL 0.1% formic acid supplemented with 0.015% N-Dodecyl-B-D-Maltoside (DDM) and mix at 1800 rpm, Room temperature , 00:30:00 (ThermoMixer C, Eppendorf).

30m

25 If analysing immediately, transfer 20 µL of each sample to a 96-well plate or LC-MS vials and inject 200 ng peptides per sample for MS analysis.

Offline HPLC Fractionation of Pooled Sample Peptides for Spectral Library Generation

26 Aliquot 20 µL from each sample and pool in a 2 mL Protein LoBind Eppendorf tube (Sarstedt).

> Note
>
> Depending on sample numbers, this volume may need to be increased or decreased.

27 Transfer to an LC-MS vial/plate and inject 50% of pooled peptides (approximately 44 µg) into a Vanquish HPLC system inline with a Fraction Collector using system settings.

Time	Flow [ml/min]	%B
0	Equilibration	
0	Run	
0	0.275	3.000
19	0.275	3.000
20	0.400	10.000
50	0.400	40.000
55	0.400	60.000
57	0.400	95.000
60	0.400	95.000
61	0.400	3.000
70	0.400	3.000
70.1	0.010	3.000

Solvent A – 10mM Ammonium Formate (pH 10)

Solvent B – 10mM Ammonium Formate (pH 10), 80% Acetonitrile

All changes are linear: Curve = 5

New fraction collected every 30 seconds starting at 21 minutes, directly into a LoBind twintec 96-well plate (Eppendorf).

28 Following fraction collection, vacuum centrifuge samples at Room temperature until dry and store at -20 °C until mass spectrometry analysis.

Note

Samples are dried and later resuspended in the original 96-well LoBind collection plate to minimise peptide losses through sample transfer, and all fractions are additionally subsequently injected directly from each well for MS analysis. Ensure plate is tightly sealed before storing at -20 °C.

Spectral Library Generation using Narrow-Window Data-Independent Acquisition Mass Spectrometry

32m

29 Centrifuge dried peptides (contained within LoBind 96-well plate) for 54 rcf, 00:01:00 (Centurion Scientific). 1m

30 Add 20 µL of 0.1% formic acid supplemented with 0.015% N-Dodecyl-B-D-Maltoside (DDM) and mix at 500 rpm, Room temperature , 00:30:00 (ThermoMixer C, Eppendorf). 30m

> Note
>
> To prevent potential sample loss, following addition of resuspension buffer, reseal the LoBind plate prior to shaking.

31 Centrifuge resuspended peptides for 54 rcf, 00:01:00 (Centurion Scientific). Remove plate seal. 1m

32 Inject 10 µL of each fraction into a Vanquish Neo UHPLC system inline with an Orbitrap Astral mass spectrometer for DIA-MS analysis.

33 Trap and elute peptides using an Acclaim™ PepMap™ 100 C18 HPLC column (3 µm particle size, 75 µm diameter, 150 mm length) and separate using an EASY-Spray™ PepMap™ Neo UHPLC column (2 µm C18 particle, 75 µm diameter,150 mm length) with LC conditions.

Buffer A: 0.1% formic acid
Buffer B: 80%ACN, 0.1% formic acid

Time	Duration (Min)	Flow [µl/min]	%B	Volume [µl]	No. of Column Volumes
0	Run				
0	0	1.3	4	0	0
0.5	0.5	1.3	4	0.65	0.37
0.6	0.1	0.8	8	0.11	0.06
0.9	0.3	0.8		0.24	0.14
13.9	13	0.8	22.5	10.4	5.86
20.8	6.9	0.8	35	5.52	3.11
21.2	0.4	2	55	0.56	0.32
21.2	Column Wash				
21.7	0.5	2	99	1	0.56
22.6	0.9	2	99	1.8	1.01
22.6	Stop Run				
22.6	Column Equilibration				

34 Acquire data using MS settings.

	A	B
	Global Parameters	
	Application Mode	Peptide
	Method Duration (min)	22.6
	Settings	
	Infusion Mode	Liquid Chromatography
	Expected LC Peak Width (s)	10
	Advanced Peak Determination	☑
	Default Charge State	2
	Orbitrap Lock Mass Correction	Off
	Ion Source Properties	

	A	B
	Ion Source Type	NSI
	Spray Voltage	Static
	Positive Ion (V)	1900
	Negative Ion (V)	600
	Ion Transfer Tube Temp (°C)	290
	Use Ion Source Settings from Tune	☐
	FAIMS Mode	Not Installed
	Full Scan Properties	
	Orbitrap Resolution	240000
	Scan Range (m/z)	380-980
	RF Lens (%)	40
	AGC Target	Custom
	Normalised AGC Target (%)	500
	Maximum Injection Time (ms)	5
	Microscans	1
	Data Type	Profile
	Polarity	Positive
	Source Fragmentation	☐
	DIA MS Settings	

	A	B
	Start Time (min)	0
	End Time (min)	22.6
	Precursor Mass Range (m/z)	380-980
	DIA Window Type	Auto
	Isolation Window (m/z)	4
	Window Overlap (m/z)	0
	Window Placement Optimisation	On
	Number of Scan Events	149
	DIA Window Mode	m/z Range
	Collision Energy Type	Normalised
	HCD Collision Energy (%)	25
	Detector Type	Astral
	TMT	Off
	Scan Range (m/z)	150-2000
	RF Lens (%)	40
	AGC Target	Custom
	Normalized AGC Target (%)	500
	Absolute AGC Value	5.000e4
	Maximum Injection Time (ms)	3

	A	B
	Microscans	1
	Data Type	Centroid
	Polarity	Positive
	Source Fragmentation	Disabled
	Loop Control	Time
	Time (sec)	0.6

Spectral Library Generation using DIA-NN Software (version 1.8.1)

35 Generate a predicted spectral library in DIA-NN by providing the downloaded reviewed (SwissProt) human UniProt FASTA file, searched with the following parameters:

Command

new command name

```
diann.exe --lib  --threads 24 --verbose 1 --out C:\Database\20230102-
Libraries\20230102_UniprotSwissProt_Human_Cano+Iso - Ox+Ac --qvalue
0.01 --matrices --out-lib C:\ Database\20230102-
Libraries\20230102_UniprotSwissProt_Human_Cano+Iso - Ox+Ac --gen-spec-
lib --predictor --fasta
C:\Database\20230102_UniprotSwissProt_Human_Cano+Iso.fasta --fasta-
search --min-fr-mz 200 --max-fr-mz 1800 --met-excision --cut K*,R* --
missed-cleavages 1 --min-pep-len 7 --max-pep-len 30 --min-pr-mz 300 --
max-pr-mz 1800 --min-pr-charge 1 --max-pr-charge 4 --unimod4 --var-
mods 1 --var-mod UniMod:35,15.994915,M --var-mod
UniMod:1,42.010565,*n --monitor-mod UniMod:1 --reanalyse --relaxed-
prot-inf --smart-profiling --peak-center --no-ifs-removal
```

36 Use this newly created spectral library to search the 96 peptide fractions, enabling the generation of a new spectral library from the search output:

Command

new command name

```
diann.exe --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F96.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F95.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F94.raw  --f \\MRC-
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ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F12.raw  --f \\MRC-
```

```
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F11.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F10.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F9.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F8.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F7.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F6.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F5.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F4.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F3.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F2.raw  --f \\MRC-
ASTRAL\DataSSD\2024\July\20240711\20240713_MJ_Skin_F1.raw  --lib
\\Mrc-astral\d\Share\Matthew\20230102_UniprotSwissProt_Human_Cano+Iso
- Ox+Ac.predicted.speclib --threads 64 --verbose 1 --out
D:\Users\Matt\Skin_PPMI\SpecLib\report.tsv --qvalue 0.01 --matrices --
temp D:\Users\Matt\Skin_PPMI\SpecLib --out-lib
D:\Users\Matt\Skin_PPMI\SpecLib\Skin_pilot_speclib.tsv --gen-spec-lib
--var-mods 1 --var-mod UniMod:35,15.994915,M --individual-mass-acc --
individual-windows --smart-profiling --peak-center --no-ifs-removal
```