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Sample preparation protocol for proteomic analysis of isolated lysosomes and whole cell extracts V.1

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We use this protocol and it's working

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Abstract

Mass spectrometry-based proteomics has emerged as fundamental technique to study functional changes of proteome including post translational modifications. Sample preparation is key for an effective and reproducible identification and quantification for proteomic analysis. Here, we describe a step wise protocol for samples derived from cell lines models or isolated human cells. The protocol has been optimised for organelle pulldown preps. To maximize proteomic coverage, we deploy a strong detergent (2% SDS), as well as high energy sonication to ensure complete solubilization of tissue/cellular proteins. We describe a facile protocol for straightforward capture of solubilized protein samples on a S-trap column that allows removal of SDS and other components that interfere with protease digestion. We provide an optimized trypsin/Lys-C protease digestion protocol to maximize protein digestion.

Attachments

**1015-2623.pdf**

104KB

Guidelines

It is recommended to have 1:10 ratio of trypsin e.g. for 10ug of protein you would supplement with 1ug of Trypsin + Lys C. For S-Trap micro columns, it is recommended to have at least 1ug of trypsin irrespective of sample amount i.e. for anything 10ug less starting material.



Materials

Reagents:

- Milli-Q H₂O
-  cOmplete™ EDTA-free Protease Inhibitor Cocktail **Merck MilliporeSigma (Sigma-Aldrich) Catalog #11873580001**
-  Roche PhosSTOP™ **Merck MilliporeSigma (Sigma-Aldrich) Catalog #4906837001**
-  Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T7408-100ML**
-  Pierce BCA Protein Assay Kit **Thermo Fisher Scientific Catalog #23225**
-  Tris(2-carboxyethyl)phosphine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #75259**

Note

Prepare and store 10 µl aliquots of 1 Molarity (M) TCEP in Milli-Q H₂O. Prior to use dilute the 1 Molarity (M) TCEP solution 10 x in 300 millimolar (mM) TEABC to generate a stock solution of 0.1 Molarity (M) TCEP in 300 millimolar (mM) TEABC.

-  Pierce™ Trypsin/Lys-C Protease Mix, MS-Grade **Thermo Fisher Catalog #A40007**
-  Acetonitrile ≥99.9% **VWR International Catalog #1.00030.2500**
-  LC-grade Formic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #695076**
-  Trifluoroacetic acid for HPLC > 99.0% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #302031-100ML**


Note

Prepare and store 20 % (by vol) aqueous TFA stock at 4 °C.

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

Note

Prepare an aqueous 200mM stock solution iodoacetamide just before use

-  Methanol LiChrosolv® hypergrade for LC-MS Supelco® **VWR International Catalog #1.06035.2500**
- HEPES: 200mM aqueous solution of HEPES pH 8 used as a stock

- SDS: 20% (by mass) solution of SDS used as a stock
- Lysis Buffer: 20mM aqueous HEPES solution with 2% SDS supplemented with protease and phosphatase tablets

A	B
HEPES	20mM
SDS	2%

- S-Trap Wash Buffer: 90 % (by vol) aqueous LC grade methanol containing a final concentration of 100 millimolar (mM) TEABC made from a 1 Molarity (M) TEABC stock

A	B
Methanol	90%
TEABC	100 millimolar (mM)

S-Trap Elution Buffer 1

50 mM TEABC in LC-MS water



S-Trap Elution Buffer 2

0.15% (by vol) LC-MS grade formic acid in LC-MS water

S-Trap Elution Buffer 3

Aqueous solution of 80% acetonitrile and (by vol) and 0.15% formic acid

Equipments:

-  DynaMag™- Spin Magnet **Thermo Fisher Catalog #12320D**
-  Bioruptor Plus sonication system **Diagenode Catalog #B01020001**

Equipment

Eppendorf ThermoMixer

NAME

Eppendorf ThermoMixer® C

BRAND

EP02095

SKU

<https://www.camlab.co.uk/eppendorf-thermoixer-c>

LINK

Equipment

ThermoTop®

NAME

Smart block

TYPE

Eppendorf

BRAND

5308000003

SKU

Set of gilson pipettes P10, P200, P1000

Multichannel pipette 20- 100 µL

Plate reader for Protein quantification (BioTek Epoch)







Benchtop centrifuge (VWR)



Savant™ SpeedVac™ Medium Capacity Vacuum Concentrators for Combinatorial Chemistry Applications **Thermo Fisher Catalog #SPD140DDA-115**

Consumables:




-  SafeSeal reaction tube 1.5 ml PP PCR Performance Tested Low protein-binding **Sarstedt Catalog #72.706.600**
-  Protein LoBind® Tubes **Eppendorf Catalog #0030108132**
-  PIPETTE TIPS 100- 1000 µL BLUE SUITABLE FOR EPPENDORF STERILE 60 PIECES PER RACK **greiner bio-one Catalog #686271**
-  PIPETTE TIP 10 - 100 µL SUITABLE FOR EPPENDORF 96 PIECES / ST RACK **greiner bio-one Catalog #685261**
-  S-Trap™ micro columns (≤ 100 µg) **Protifi Catalog #C02-micro**
-  Microplate, 38-well, PS, F-Bottom **greiner bio-one Catalog #781101**






Sample lysis and elution of lysosomal material - For immunoprecipitates:

16m 30s

- 1 Resuspend your dry bead slurry of LysoTag or MockTag IP in  100 μ L of HEPES lysis buffer, making sure to disperse any clumps.


Note

The IP prep can be made on the day following the procedure outlined here: dx.doi.org/10.17504/protocols.io.x54v9yp51g3e/v1 or you can use IP-preps stored at  -80 °C .

- 2 Incubate on  Room temperature for  00:15:00 .

15m





- 3 Place the tubes on a tube magnet for  00:00:30 .

30s

- 4 Pipette the supernatant to a fresh 1.5ml Eppendorf tube.






- 5 Sonicate samples using a Diagenode Bioruptor (use it at high energy for 15 cycles ( 00:00:30 ON/  00:00:30 -Off).

1m

For whole cell samples:

26m

- 6 Resuspend the pellet in  100 μ L of lysis buffer, making sure to disperse any clumps.

- 7 Incubate on  Room temperature for  00:15:00 .

15m



- 8 Centrifuge at  17000 x g for  00:10:00 .

10m





9 Pipette the supernatant to a fresh 1.5ml Eppendorf tube.



10 Sonicate samples using a Diagenode Bioruptor (use it at high energy for 15 cycles (

00:00:30 ON/ 00:00:30 -Off)

1m

Protein Quantification

30m

11 Create protein standards using BCA Protein Assay Kit BSA solution (1500, 1000, 750, 500, 250, 125, 62.5, 31.25, 16, 125, 0 ng / μ L).

Note

Dilute the BSA solution with your Lysis Buffer

12 In a 384-well plate, pipette 5 μ L of your sample and standards into wells in duplicates.



Note

For enhanced sensitivity of lower range of concentrations, using a ratio of 1 : 8 sample to BCA reagent on a 384-well plate allows for more accurate representation of lower protein concentrations for IP-preps. Additionally, you might need to dilute your whole cell samples by a factor of 2-5 depending on the cell type (eg. tissues from mice lysed in 100 μ L might have higher concentration than the standards 1500 ng/ μ L).

13 Mix your BCA Reagent A and B at ratio of 50 :1.



14 Using a multichannel pipette, add 40 μ L of your BCA reagent mix (Step 13) to each of the wells that contain your samples/standards.



**Note**

Avoid making bubbles as this will influence the readings you get.

15 Incubate in 37 °C for 00:30:00 .

30m



16 Record the 562nm absorbance of your plate.

17 Calculate the concentration of your samples using your standard curve.

Processing for peptide digestion

1h 30m

18 Make your samples the same concentration in fresh 1.5ml Eppendorf tubes.

Note

This is to standardise the amount of protein to be digested. Your standardisation reference should be the concentration of your LysoTag-IP sample. It is important to digest the same amount of LysoTag-IP and whole cell samples. Remember that your MockTag-IP samples might have barely any protein in them and for these samples, do process everything you have.

19 Add 5 millimolar (mM) TCEP to reduce your sample and incubate on a Thermomixer for 00:30:00 at 60 °C and 1350 rpm .

30m



20 Cool the sample and the Thermomixer to 25 °C .

21 Add 20 millimolar (mM) IAA to your sample and incubate Thermomixer for 00:30:00 at 25 °C and 1350 rpm .

30m





22 Quench alkylation by adding [1M] 5 millimolar (mM) TCEP and incubate on a Thermomixer for

00:30:00 at 25 °C and 1350 rpm .

30m



23 Supplement with additional SDS to achieve final 5% SDS to your sample and mix well by flicking.



24 Add 1% TFA.



25 Add 6x the current volume of S-Trap Wash Buffer and mix well.



Loading onto a S-Trap micro column

4m

26 Prepare a separate set of 2ml Eppendorf tubes and insert S-Trap micro columns inside them.

27 Pipette 200 µL of your sample (Step 25) to the column.



28 Centrifuge at 1000 x g for 00:01:00 to capture the protein particles onto the column.

1m



29 Repeat steps 28 and 29 until you run out of your sample.

Note

You will need to empty the flowthrough in the 2ml Eppendorf tubes before the flowthrough reaches the S-Trap column bottom.

30 Pipette 160 µL of fresh S-Trap Wash Buffer into the column.



31 Centrifuge at 1000 x g for 00:01:00 .

1m





32 Repeat steps 31 and 32 twice more.

32.1 Centrifuge at  1000 x g for  00:01:00 (1/2)

1m



32.2 Centrifuge at  1000 x g for  00:01:00 (2/2)

1m



33 Take the column and transfer it to a new 1.5ml Eppendorf tube.

Trypsin + Lys C Digestion of the column




1d 1h



34

Note

It is recommended to have 1:10 ratio of trypsin e.g. for 10µg of protein you would supplement with 1ug of Trypsin + Lys C. For S-Trap micro columns, it is recommended to have at least 1ug of trypsin irrespective of sample amount i.e. for anything 10µg less starting material.

Dissolve Trypsin + Lys C in  50 millimolar (mM) TEABC.

34.1 Dissolve the Trypsin + Lys C to the desired concentration based on the amount of protein digested. Note that the S-Trap Micro Columns only holds up to  150 µL of liquid. For optimal results, aim to add  40 µL -  80 µL of your mix from step 34.

35 Add  40 µL -  80 µL of the Trypsin + Lys C mix (Step 34) and add it inside the column.



Note

Do not touch and disturb the actual resin membrane inside the column. Additionally, avoid any bubbles from forming inside the column.



36 Screw the lid on the column loosely.

37 Incubate for 01:00:00 at 25 °C without agitation and then 24:00:00 at 47 °C without agitation.

1d 1h



Elution from the column

4m

38 Add 60 µL of [M] 50 millimolar (mM) TEABC.



39 Centrifuge at 1000 x g for 00:01:00 .

1m



40 Add 60 µL 0.15% formic acid.



41 Centrifuge at 1000 x g for 00:01:00 .

1m



42 Remove the column and place it in a fresh 1.5ml Eppendorf tube.

Note

Keep the original tube with the flowthrough from steps 38-41

43 Add 60 µL of Elution Buffer to the column.






44 Centrifuge at 1000 x g for 00:01:00 .

1m



45 Repeat steps 43 and 44.



- 45.1
- Add  60 μ L of Elution Buffer to the column.
 - Centrifuge at  1000 x g for  00:01:00 .

1m



46 Pool your samples from steps 45 and 41.

47 Vacuum dry your samples and store at  -80 °C .