Sample preparation protocol for total proteomic analysis of mouse tissues

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Protocol status: Working

We use this protocol as a standard in our lab to prepare total proteomic analysis of cells and tissues.

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Sample preparation protocol for total proteomic analysis of mouse tissues including brain V.1

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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 tissue specimens derived from human/mice tissue and cell lines models. 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. We also explain how to undertake tandem mass tag labelling of digested proteins to multiplex up to 16 samples in a single study. We also provide an notes of caution for each step of the sample preparation as well as providing a comprehensive list of all of the required reagents and apparatus. Using this protocol, we achieve a high depth of proteome coverage by identifying >10,000 protein groups reproducibly in all tissues and cells we have analyzed. We believe our protocol can be easily adopted and implemented in the routine large-scale proteomic analysis of cells/tissues extracts to achieve high depth and reproducible protein analysis.

ATTACHMENTS

[Sample_preparation_protocol_for_total_proteomic_analysis_of_mouse_tissues_including_brain.pdf](#)

MATERIALS

Consumables

- Marker pen
- Pipette set (1 ml, 200 µl, 100 µl, 20 µl, 10 µl)
- PPE kit (Lab coat, gloves, safety glasses)
- Dry ice
- Liquid Nitrogen
- Ice bucket
- 1.5 ml eppendorf tubes rack
- PTFE-O rings (To place the stage-tip into the Eppendorf tubes. Generally, you could get from NEST group desalting columns and re-use them <https://www.nestgrp.com/>)
- S-Trap mini columns (<https://www.protifi.com/>)

Equipment	
Protein LoBind® Tubes	NAME
Eppendorf	BRAND
022431081	SKU
https://online-shop.eppendorf.us/US-en/Laboratory-Consumables-44512/Tubes-44515/Protein-LoBind-Tubes-PF-56251.html	LINK

Equipment	
Pipette tips low binding (1 ml, 250 µl, 10 µl)	NAME
Star Lab	BRAND
S1111-3700, S1111-1706, S1111-6700	SKU
https://www.starlabgroup.com/en/consumables/pipette-tips_WebPSub-155853/10-l-graduated-tip-refill_SLS1111-3700.html	LINK

Equipment	
96 well plate- clear	NAME
Greiner Bio-one	BRAND
655101	SKU
https://shop.gbo.com/en/usa/products/bioscience/microplates/96-well-microplates/96-well-microplates-clear/655101.html	LINK

Equipment	
2 ml tubes	NAME
Axygen	BRAND
MCT2000	SKU
https://ecatalog.corning.com/life-sciences/b2c/US/en/General-Labware/Tubes/Microcentrifuge-Tubes/Axygen%C2%AE-MaxyClear-SnapLock-Microcentrifuge-Tubes/p/MCT-200-O	LINK

Equipment	
16-gauge needle	NAME
Sigma Aldrich	BRAND
Z261378	SKU
https://www.sigmaaldrich.com/catalog/product/aldrich/z261378?lang=en&region=US	LINK

Equipment	
spray duster	NAME
Q-connect	BRAND
KFO4499	SKU
https://q-connect.com/products/cleaning/spray-dusters/hfc-free-flammable-spray-duster-400ml	LINK

Equipment	
X100 20 mL Amber Glass EPA Vial	NAME
Cole Parmer	BRAND
11533750	SKU

Equipment	
X72 40 mL Amber Glass EPA vial W Cap and seal	NAME
Cole Parmer	BRAND
10572553	SKU

Note
Note: Prepare all stock and working reagents in these amber vials to store as per the protocol.

Equipment	
pH strips	NAME
Millipore	BRAND
1.09584.0001	SKU
https://us.vwr.com/store/product/31936942/mquant-ph-test-strips-milliporesigma	LINK

Equipment	
Sep-Pak Vac 1cc (50 mg) tC18-Cartridges	NAME
Waters	BRAND
WAT054960	SKU
https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/wat054960-sep-pak-tc18-1-cc-vac-cartridge-50-mg-sorbent-per-cartridge-37-.html	LINK

Equipment	
XBridge BEH C18 Column, 130A, 3.5 um, 4.6 x 250 mm	NAME
Waters	BRAND
186003943	SKU
https://www.waters.com/nextgen/us/en/shop/columns/186003943-xbridge-beh-c18-column-130a-35-m-46-mm-x-250-mm-1-pk.html	LINK

Equipment	
96 well 2 ml deep well plates	NAME
BRAND	BRAND
10680763	SKU
https://www.fishersci.co.uk/shop/products/brandtech-brand-deep-well-plates-4/10680763	LINK

Equipment	
Acclaim pepmap 100 100um*cm nano viper trap column	NAME
Thermo Scientific	BRAND
11312263	SKU
https://www.fishersci.co.uk/shop/products/acclaim-3-m-pepmap100-nano-lc-columns-nanoviper-fittings/11312263	LINK

Equipment	
Easy-Spray PepMap RSLC C18 2um, 50cmx75um	NAME
Thermo Fisher Scientific	BRAND
ES802	SKU
https://www.fishersci.com/shop/products/thermo-scientific-easy-spray-columns-particle-size-2-m-250-x-0-075-mm-i-d/03052573	LINK

Reagents

- SDS Lysis Buffer: Final [M] 2 % (by mass) SDS in [M] 100 millimolar (mM) Triethylammonium bicarbonate $\text{pH } 8.5$ (TEABC, this is the natural pH of this buffer and made from a [M] 1 Molarity (M) TEABC stock purchased from Sigma Catalogue number #T7408-500 mL), [M] 1 millimolar (mM) sodium orthovanadate, [M] 50 millimolar (mM) NaF, [M] 10 millimolar (mM) b-glycerophosphate, [M] 5 millimolar (mM) sodium pyrophosphate, [M] 1 Mass Percent microcystin-LR, and complete EDTA-free protease inhibitor cocktail (Roche)

Triethylammonium bicarbonate buffer (TEABC) **Sigma Aldrich Catalog #T7408**

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

- Tris (2-carboxyethyl) phosphine (TCEP) **Sigma Aldrich Catalog #75259-10G**

Note

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

- Ortho-Phosphoric acid 85% (by vol) **Sigma Aldrich Catalog #5438280100**

Note

Note: Prepare [M] 12 % (by vol) stock aqueous phosphoric acid by diluting in water and store in 4 °C.

- S-Trap protein binding buffer ([M] 90 % (by vol) aqueous LC grade methanol containing a final concentration of [M] 100 millimolar (mM) TEAB, 7.1, made from a [M] 1 Molarity (M) TEABC stock purchased from Sigma Aldrich #T7408-500 mL)

- Sequencing Grade Modified Trypsin (5 X 20 ug pack) **Promega Catalog #V5111**

Note

Note: Store stocks in -20 °C freezer and thaw trypsin stock just before the digestion step.

- Methanol LiChrosolv® hypergrade for LC-MS **VWR international Ltd Catalog #1.06035.2500**
- Acetonitrile ≥99.9% LiChrosolv® Reag. Ph. Eur. gradient grade for liquid chromatography **VWR international Ltd Catalog #1.00030.2500**
- Iodoacetamide **Millipore Sigma Catalog #I1149**
- LC grade Formic acid **Sigma Catalog # 695076**

- Trifluoroacetic acid **Sigma Aldrich Catalog #302031**

Note

Note: Prepare and store **1M** 20 % (by vol) aqueous TFA stock at **4 °C**.

- Empore C18 disks 47 mm **Cole Parmer Catalog #2215**

Note

Note: Prepare a single layer with 16-gauge needle and pass it with spray duster into the 250 µl tip for 0.1 to 5 µg of peptide amount. For more than 5 µg use 2 or 3 layers of C18 material. Refer Figure 1 -see below for Stage-tip assembly.

- 11 plex TMT-Kit (Thermo Fisher Scientific# 90110 and A37725) or 16 plex TMT-Kit (Thermo Fisher Scientific #A44520) depending on number of samples being analysed) (

TMT10plex Isobaric Label Reagent Set 1 x 0.8 mg **Thermo Fisher Scientific Catalog #90110**

or

TMT10plex Isobaric Label Reagent Set plus TMT11-131C Label Reagent **Thermo Fisher Catalog #A37725**

or

TMTpro™ 16plex Label Reagent Set 1 x 5 mg **Thermo Fisher Scientific Catalog #A44520**

)

- Anhydrous Acetonitrile **Sigma Aldrich Catalog #271004**
- 50% (by vol) Hydroxylamine by mass **Sigma Aldrich Catalog #467804**
- [M] 20 % (by mass) aqueous SDS stock
- LC buffer ([M] 0.1 % (by vol) Formic acid in [M] 3 % (by vol) Acetonitrile)
- Solvent-A1 ([M] 0.1 % (by vol) TFA)
- Solvent-A2 ([M] 0.1 % (by vol) Formic acid)
- Solvent-B1 ([M] 50 % (by vol) acetonitrile , [M] 0.1 % (by vol) TFA)
- Solvent-B2 ([M] 60 % (by vol) acetonitrile , [M] 0.1 % (by vol) Formic acid)

Equipment

- Pulveriser kit (<https://cellcrusher.com/>)
- -80 °C deep freezer, -20 °C freezer and 4 °C fridge
- Benchtop centrifuge (VWR)
- Milli-Q water system
- Orbital shaker
- pH meter
- Plate reader for Protein quantification (BioTek Epoch)
- Diagenode Bioruptor plus sonication system
- Eppendorf Thermomixer
- 1.5 ml tube floaters
- Branson water bath sonicator
- Dionex RSLC 3000 nano-LC system
- Dionex RSLC 3000 LC system for Offline fractionation with Auto sampler or Fraction collector, micro pump and VWD detector
- Orbitrap Fusion Lumos Tribrid Mass spectrometer
- Nanodrop 1000 (Thermo Fisher Scientific)

Equipment	
Savant Speedvac system	NAME
Thermo Fisher	BRAND
SPD140DDA	SKU
https://www.thermofisher.com/order/catalog/product/SPD140DDA-115#/SPD140DDA-115	LINK

Equipment	
Rubber Pipette Bulb	NAME
Fisherbrand	BRAND
12446180	SKU
https://www.fishersci.fi/shop/products/latex-dropper-teats/12446180	LINK

SAFETY WARNINGS

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

Sample preparation and S-Trap Assisted Digestion

- 1 Snap freeze mouse tissue immediately after isolation in liquid nitrogen and store at -80°C .
- 2 Pulverize frozen tissue in liquid nitrogen to a fine powder and snap freeze immediately in 2 ml tubes. This is done using cell crusher kit.

Note

Note: Maintain all Pulverizer units in liquid nitrogen including spatula. After use wash the pulveriser with a tap water and clean thoroughly several times with 70% (by vol) ethanol.
- 3 Weigh pulverized tissue powder on a weighing balance (5-7% of the weight should be protein i.e. ~ 5 to 7 mg protein/100 mg).

Note

Note: Keep samples on dry ice during after weighing. Take a 1.5 ml tube, check the weight on weighing balance and subsequently tare the weight. Now take small scoop of sample using pre-chilled fine spatula to weigh the sample).

- 4 Immediately add *10 times* of the weight of the tissue of **1M 2 % (by mass) SDS lysis buffer** .

Note

Note: For **100 mg tissue powder** please add **1000 µL lysis buffer** .

- 5 Put samples on an orbital shaker in cold room at **1000 rpm, 00:15:00** .

- 6 Boil samples at **95 °C** for **00:10:00** and allow to cool.

10m

- 7 Sonicate samples using a Diagenode Bioruptor (use it at high energy for 10 cycles (30Sec-ON/30Sec-Off)).

- 8 Clarify lysate by centrifuging at **20800 x g, 4°C, 00:30:00** .

- 9 Measure protein amount using the Bicinchoninic acid assay (BCA) method in triplicate at 1 to 10 and 1 to 20 dilutions - repeat analysis if readings are not close.

10 Take 200 µg protein for total proteomic analysis.

11 Perform reduction by adding a 1 in 10 dilution of a solution of 0.1 Molarity (M) TCEP dissolved in 300 millimolar (mM) TEABC to bring final concentration of TCEP to 10 millimolar (mM) .

12 Incubate on a Thermomixer for 00:30:00 at 60 °C with a gentle agitation at 1000 rpm, 23°C .

30m

13 Bring tubes to Room temperature and add a one in 10 dilution of freshly prepared 0.4 Molarity (m) iodoacetamide dissolved in water.

Note

Note: It is critical that the samples are at Room temperature prior to addition of iodoacetamide.

14 Incubate in dark on a Thermomixer at Room temperature for about 00:30:00 with a gentle agitation 1000 rpm, 23°C .

30m

15 Quench alkylation by addition of a 1 in 10 dilution of 0.1 Molarity (M) TCEP dissolved in 300 millimolar (mM) TEABC to bring final concentration of TCEP to 10 millimolar (mM) .

16 Incubate on a Thermomixer for 00:20:00 at Room temperature with a gentle agitation 1000 rpm .

20m

- 17 Add SDS to a final concentration of 5 % (by mass) from 20 % (by mass) SDS stock .

Note

Note: The lysate is already in 2 % (by mass) SDS so supplement with a stock of 20 % (by mass) SDS in order to bring the final SDS concentration to 5 % (by mass) . A final concentration of 5% SDS is recommended for complete solubilization of proteins for S-Trap assisted protocol.

- 18 Add a 1 in 10 dilution of 12 % (by vol) phosphoric acid into the sample to make a final concentration of ~ 1.2 % (by vol) phosphoric acid .

Note

Note: For example, 5 μ L to 50 μ L .

- 19 Dilute the sample to in 7 times the current volume of the mixture in of S-Trap wash buffer (90 % (by vol) methanol in 0.1 Molarity (m) TEAB pH 7.1 v/v) (for examples if sample volume is 50 μ L add 300 μ L S-Trap wash buffer ((90 % (by vol) methanol in 0.1 Molarity (m) TEAB pH 7.1 v/v)).

Note

Note: If the lysate volume is now more than 50 μ L , scale S-Trap wash buffer accordingly i.e. 7 times the volume of the lysate.

- 20 Prepare an S-Trap mini column in a 2 ml tube.

- 21 Add the diluted protein mixture to the column.

22 Centrifuge briefly to capture the protein particles. 1000 x g, 23°C

Note

Note: All subsequent wash steps needs to be done at room temperature (23°C). If you observe clogging or if the sample doesn't pass through the column then increase centrifugation speed up to a maximum of 4,000g.

23 Wash column with 400 µL S-Trap buffer a total of 4 times (spin 1000 x g, 00:01:00 between washes).

Note

Note: The protein remains bound on the column and SDS and buffer components that effect trypsin digestion are removed.

24 Move the S-Trap column to a clean 2 ml tube for digestion.

25 Add a 100 µL solution of freshly dissolved trypsin containing 13 µg trypsin and 2 µg Lys-C freshly dissolved in [mM] 100 millimolar (mM) TEAB (1:15).

Note

Note: We use 6.5 µg trypsin and 1 µg Lys-C per 100 µg protein .

26 Centrifuge briefly at 200 x g, 00:01:00 .

27 Collect flowthrough and reapply the trypsin solution back onto the column being careful to avoid air bubbles.

28 Cap the tubes and incubate at 47 °C *without shaking* for 01:30:00 . 1h 30m

Note

Note: **Do not shake** as this causes bubbles and damages the column.

29 Incubate for another 02:00:00 at Room temperature . 2h

30 Add 80 µL 50 mM TEAB then spin to elute and place the eluate in a new 1.5 ml Eppendorf tube termed "eluate tube".

31 Next, add 80 µL 0.15% (by vol) Formic Acid and spin to elute. Also add this eluate to the "eluate tube".

32 Finally, add 80 µL 50% (by vol) Acetonitrile in 1M 0.15 % (by vol) formic acid and spin to elute. Also add this eluate to the "eluate tube".

Note

Note: 3 eluates should have been added to the eluate tube.

33 Take 1 µL - 2 µL combined eluate , vacuum dry and inject on MS to verify the digestion efficiency.

Note

Note: Analyse data with a 01:10:00 gradient run on QE HF-X or Orbitrap Lumos mass spectrometer in a FT-FT-HCD mode. Search data with Proteome Discoverer 2.1 or 2.4 version. Determine the digestion efficiency by plotting number of missed cleavages. Zero missed cleavages should be >75% and single missed cleavages should be between 20-23%.

- 34 Vacuum dry the remaining peptide amount and store in -80 °C deep freezer until ready to undertake TMT labelling.

Tandem Mass Tags Labelling

- 35 Dissolve 800 µg of each of the TMT mass tag reagents within the 11 or 16-plex TMT reagent kit with 41 µL 100% by vol anhydrous acetonitrile to obtain a 20 µg/µL concentration for each TMT reporter tag.

- 36 Leave them at Room temperature for 00:10:00 . Following, vortex and briefly spin 2000 x g, 00:02:00 .

10m

Note

Note: Dissolved TMT reagents are prone to hydrolysis so immediately after aliquoting store remainder reagent in -80 °C deep freezer for long-term storage up to six months and try to avoid multiple freeze thaw cycles.

- 37 Dissolve lyophilized peptides in 50 µL of a mixture containing 38 µL 50 mM TEAB buffer + 8 µL 100% (by vol) anhydrous acetonitrile .

Note

Note: It is important to maintain a final 30 % (by vol) of anhydrous Acetonitrile for an effective TMT reaction.

38 Keep samples on a floater and place it on a water bath sonicator for 00:10:00 . 10m

39 Centrifuge samples at high speed 20800 x g, 00:10:00 , room temperature .

40 Transfer dissolved peptides into a 1.5 ml protein low-binding eppendorf tube.

41 Add 10 μ L 20 μ g/ μ L TMT reagent i.e. 200 μ g aiming for a 1:1 mass ratio of peptide:TMT reagent.

42 Give a gentle vortex and brief spin 2000 x g, 00:01:00 .

43 Place samples on a Thermomixer and incubate with a gentle agitation at 800 rpm, 02:00:00 room temperature .

44 Add another 50 μ L 50 mM TEAB buffer to make a final 100 μ L reaction. Vortex, brief spin at 2000 x g, 00:01:00 and incubate on a Thermomixer for 00:10:00 . 10m

Note

Note: It is a good practice to maintain the total volume to 100 μ L final reaction as it helps in reducing pipetting error when aliquoting 5 μ L of sample for label check efficiency).

- 45 In order to verify the TMT labelling efficiency of each TMT mass tag, take a 5 µL aliquot from each of the TMT samples and pool this in a single tube and vacuum dry immediately using a SpeedVac.

Note

Note: It is important to verify the labelling efficiency of each TMT mass tag and it should label > 98%, by analysing on Mass spec. We recommend doing this employing a 02:25:00 FT-FT-MS2 study. This will establish that each reporter tag is efficiently labelled and ensure that an equal level of each peptide is labelled with each of the TMT tags. Search MS raw data with Proteome Discoverer 2.2 or 2.4 by enabling TMTreporter tag mass (+229.163 Da) on Lysine residue and Peptide N-terminus as dynamic modifications. Filter TMT labelled Peptide spectral matches (PSMs) in the modification tab to calculate the number of labelled and unlabelled PSMs to determine the labelling efficiency. Also, export PSM abundance in txt.file, to plot a Boxplot using R-software to determine the ~1:1 abundance within and between replicates.

- 46 Place remaining 95 µL of the reaction in -80 °C freezer. If the labelling efficiency is >98% and levels of each labelled peptide appear to be close to 1:1 then proceed with the below steps.

- 47 Thaw stored TMT labelled samples from step 46 to Room temperature .

- 48 Prepare 5 % (by vol) final Hydroxyl amine solution by dissolving in water from a 50 % (by vol) stock solution .

- 49 Add 5 µL 5% (by vol) Hydroxylamine to each sample to quench TMT reaction by incubating the reaction at Room temperature on a Thermomixer for 00:20:00 .

20m

- 50 Pool all samples into a single tube.
- 51 Take 20% of the reaction i.e. **220 µL** to as a backup, snap freeze on dry ice and vacuum dry.
- Note**
- Note: This is important because if there is a sample loss during the downstream analysis or to further validate the findings.
- 52 Snap freeze the remaining **880 µL reaction** and vacuum dry using Speed vac, for high pH fractionation.
- 53 Desalt sample using tC18 50 mg cartridge by following the protocol described **73**.
- 54 After following C18 clean-up protocol, submit desalted sample to MS facility for high pH fractionation and request to fractionate to 96 fractions and concatenate by pooling distant fractions e.g A1+D1, A2+D2.. B1+E1, B2+E2 and so on to a total of 48 fractions for LC-MS/MS analysis.
- Note**
- Note: Deep fractionation significantly reduces ion interference and ratio distortion caused by co-elution of peptides in turn and improves TMT-reporter ion quantification thus increasing the reliability of peptide and protein quantifications. Also, deep fractionation greatly improves the dynamic range of peptides/proteins and increases the coverage. We generally identify between 10,000 to 12,000 quantified protein groups.
- 55 For each of the 48 fractions measure peptide concentrations using a Nanodrop 1000 spectrophotometer.

- 56 Prepare 2 µg of each fraction in 15 µL LC buffer (0.1% (by vol) formic acid in 3% (by vol) Acetonitrile) and submit each fraction to the mass spectrometry facility.
- 57 Analyse each fraction by acquiring data in FT-FT-FT (MS3) HCD mode on a Orbitrap Fusion Lumo 1h 25m
Mass spectrometer for 01:25:00 run for each fraction.

C18 Stage-tip Protocol to desalt TMT labelled samples prio...

- 58 Prepare single layer of C18 stage-tip using 16-gauge syringe needle.
- 59 Dissolve the vacuum dried peptides sample derived from go to step #45 in 80 µL solvent-A1 (0.1% (by vol) TFA) .
- 60 Add 80 µL 100% (by vol) Acetonitrile to the C18 stage-tip.
- 61 Centrifuge at 2000 x g, 00:02:00 , room temperature . Discard flow through.
- 62 Add 80 µL Solvent-A1 (0.1% (by vol) TFA) .

Note

Note: This is required to activate the C18 resin.

Note

This is required to equilibrate the C18 resin.

63 Centrifuge at 2000 x g, 00:02:00 , room temperature . Discard flow-through and repeat this step.

64 Load the acidified peptide digest to Stage-tip.

65 Centrifuge at 1500 x g, 00:05:00 , room temperature .

66 Reapply the flow through to the C18 stage-tip column and repeat this step.

67 Add 80 μ L solvent-A1 (0.1% (by vol) TFA v/v) , and centrifuge at 2000 x g, 00:02:00 , room temperature . Discard flow through. Repeat again.

68 Elute peptides absorbed to C18 column by placing the stage-tips into **new** 1.5 ml low binding tubes.

Note

Note: Using new tubes is important to avoid contamination.

69 Add 30 μ L Elution buffer (Solvent-B1 50% (by vol) acetonitrile in 0.1% (by vol) TFA) .

70 Centrifuge at 1500 rpm, 00:02:00 . Add another

30 µL Elution buffer (Solvent-B1 50% (by vol) acetonitrile in 0.1% (by vol) TFA) and repeat the centrifugation.

71 Immediately snap freeze on dry ice the eluates and vacuum dry the samples using a SpeedVac.

72 Submit to Mass spectrometry analysis as described in .

Sep-Pak Purification Protocol

10m

73 Dissolve vacuum dried pooled TMT sample from [go to step #53](#) in

500 µL solvent-A1 (0.1% (by vol) TFA) .

74 Place sample on a floater and place it in on a water bath sonicator and sonicate it for

00:10:00 .

10m

75 Centrifuge sample at 20800 x g, 00:10:00 at room temperature and transfer sample into a new 1.5 ml Eppendorf tube.

76 Check that the pH of the sample is acid >2, by pipetting 1 µL sample onto a pH strip.

Note

Note: This is important because Stage-tips are equilibrated to acidic pH and this aid in proper binding of acidified peptides to C18 resin, if sample appears to be not acidic increase TFA concentration to 0.15 % (by vol) and recheck pH.

77 Prepare single tC18 50 mg Sep-Pak cartridge and place this column in 15 ml falcon tube.

78

Add 1 mL 100% (by vol) Acetonitrile . Centrifuge on an Eppendorf centrifuge at 60 x g, 00:01:00 and discard flowthrough. Repeat this step again.

Note

Note: This step is required to activate the C18 resin.

79

Equilibrate the tC18 50 mg Sep-Pak cartridge, by adding 1 mL solvent-A1 (0.1% (by vol) TFA v/v) . Centrifuge on an Eppendorf centrifuge at 60 x g, 00:01:00 and discard flowthrough. Repeat this step two more times.

80

Load acidified sample onto the tC18 50 mg Sep-Pak cartridge.

Note

Note: Do not centrifuge and let the sample pass through by gravity or if needed push sample using rubber bulb (Fisher brand™ Rubber Pipette Bulb #12446180).

81

Collect the flow through and reapply to the column and save the flow through.

Note

Note: If you don't detect any peptides in MS, the flow through should have the peptides and this has been caused due to poor equilibration of the column.

82

Wash the tC18 50 mg Sep-Pak cartridge by addition of 1 mL solvent-A2 (0.1% (by vol) Formic acid) . Centrifuge on an Eppendorf centrifuge at 60 x g, 00:01:00 and discard flowthrough. Repeat this step two more times.

83

Place column on a new 1.5 ml Eppendorf tube.

Note

Note: Using new tubes is important to avoid contamination.

- 84** Elute the peptides from the tC18 50 mg Sep-Pak cartridge by addition of
🧪 300 µL solventB2 (60% (by vol) Acetonitrile in 0.1% (by vol) Formic acid) .

Note

Note: Do not centrifuge and let the sample pass through by gravity or if needed push sample using rubber bulb (Fisher brand™ Rubber Pipette Bulb #12446180).

- 85** Repeat elution step (step 84):

Elute the peptides from the tC18 50 mg Sep-Pak cartridge by addition of
🧪 300 µL solventB2 (60% (by vol) Acetonitrile in 0.1% (by vol) Formic acid) .

Note

Note: Do not centrifuge and let the sample pass through by gravity or if needed push sample using rubber bulb (Fisher brand™ Rubber Pipette Bulb #12446180).

- 86** Repeat elution step (step 84):

Elute the peptides from the tC18 50 mg Sep-Pak cartridge by addition of
🧪 300 µL solventB2 (60% (by vol) Acetonitrile in 0.1% (by vol) Formic acid) .

Note

Note: Do not centrifuge and let the sample pass through by gravity or if needed push sample using rubber bulb (Fisher brand™ Rubber Pipette Bulb #12446180).

- 87** Combine the 🧪 900 µL eluate and snap freeze and vacuum dry using SpeedVac.

88 Store sample in -80 °C freezer or submit to mass spectrometry facility for high pH fractionation as described in .