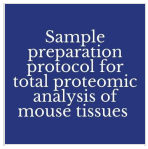


Version 1

Apr 07, 2021

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

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1 Works for me dx.doi.org/10.17504/protocols.io.bs3tngnn

Raja

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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 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

DOI

dx.doi.org/10.17504/protocols.io.bs3tngnn

PROTOCOL CITATION

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WHAT'S NEW

Usage of S-Trap columns for an effective detergent removal for on-column tryptic digestion Effective tandem mass tags (TMT) labeling for multiplexed quantitative proeomic analysis using high-resolution mass spectrometry with a minimal TMT reagents

KEYWORDS

proteomic analysis, mouse, brain, tissue, sample praparation, total proteomic analysis, proteomic

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Apr 07, 2021

PROTOCOL INTEGER ID

47955

MATERIALS TEXT

**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/)

Protein LoBind® Tubes

**Eppendorf** **022431081**

Pipette tips low binding (1 ml, 250 µl, 10 µl)

**Star Lab** **S1111-3700, S1111-1706, S1111-6700**

96 well plate- clear

**Greiner Bio-one** **655101**

2 ml tubes

**Axygen** **MCT200O**

16-gauge needle

**Sigma Aldrich** **Z261378**

spray duster

**Q-connect** **KFO4499**



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X100 20 mL Amber Glass EPA Vial

**Cole Parmer** **11533750**

X72 40 mL Amber Glass EPA vial W Cap and seal

**Cole Parmer** **10572553**

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

pH strips

**Millipore** **1.09584.0001**

Sep-Pak Vac 1cc (50 mg) tC18-Cartridges

**Waters** **WAT054960**

XBridge BEH C18 Column, 130A, 3.5 um, 4.6 x 250 mm

**Waters** **186003943**

96 well 2 ml deep well plates

**BRAND** **10680763**

Acclaim pepmap 100 100um*cm nano viper trap column

**Thermo Scientific** **11312263**



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Easy-Spray PepMap RSLC C18 2um, 50cmx75um

Thermo Fisher Scientific ES802

**Reagents**

- SDS Lysis Buffer: Final [M]2 % (by mass) SDS in [M]100 Milimolar (mM) Triethylammonium bicarbonate pH8.5 (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 Milimolar (mM) sodium orthovanadate , [M]50 Milimolar (mM) NaF , [M]10 Milimolar (mM) b-glycerophosphate , [M]5 Milimolar (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: 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 Milimolar (mM) TEABC to generate a stock solution of [M]0.1 Molarity (M) TCEP in [M]300 Milimolar (mM) TEABC .

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

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 Milimolar (mM) TEAB , pH7.1 , 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: 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**



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Iodoacetamide **Millipore**

- **Sigma Catalog #I1149**

LC grade Formic

- acid **Sigma Catalog # 695076**

Trifluoroacetic acid **Sigma**

- **Aldrich Catalog #302031**

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

Empore C18 disks 47 mm **Cole**

- **Parmer Catalog #2215**

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.

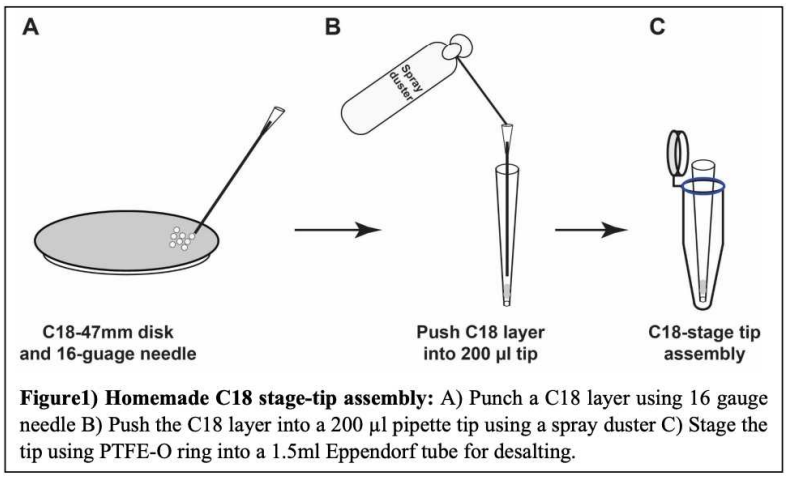


**Figure1) Homemade C18 stage-tip assembly:** A) Punch a C18 layer using 16 gauge needle B) Push the C18 layer into a 200 µl pipette tip using a spray duster C) Stage the tip using PTFE-O ring into a 1.5ml Eppendorf tube for desalting.

- 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**
- **20 % (by mass) aqueous SDS stock**
- LC buffer ( **0.1 % (by vol) Formic acid** in **3 % (by vol) Acetonitrile** )
- Solvent-A1 ( **0.1 % (by vol) TFA** )



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- 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)

Savant Speedvac system

**Thermo Fisher** **SPD140DDA**

Rubber Pipette Bulb

**Fisherbrand** **12446180**



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

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## 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: Maintain all Pulverizer units in liquid nitrogen including spatula. After use wash the pulveriser with a tap water and clean thoroughly several times with [M]**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: 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 [M]**2 % (by mass) SDS lysis buffer** .

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



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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 **[M]0.1 Molarity (M) TCEP** dissolved in **[M]300 Milimolar (mM) TEABC** to bring final concentration of TCEP to **[M]10 Milimolar (mM)** .

12  30m

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

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

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

14  30m

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

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

16  20m

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

17 Add SDS to a final concentration of **[M]5 % (by mass)** from **[M]20 % (by mass) SDS stock** .

Note: The lysate is already in **[M]2 % (by mass) SDS** so supplement with a stock of **[M]20 % (by mass) SDS** in order to bring the final SDS concentration to **[M]5 % (by mass)** . A final concentration of 5% SDS is recommended



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for complete soulblization 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: For example, **5 µl** to **50 µl** .

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 µl** add **300 µl S-Trap wash buffer** (( **90 % (by vol) methanol** in **0.1 Molarity (m) TEAB** pH **7.1** v/v)).

Note: If the lysate volume is now more than **50 µl** , 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: All subsequent wash steps needs to be done at room temperature (23$^{O}$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: 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.



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

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  1h 30m

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

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

29  2h

Incubate for another **02:00:00** at **Room temperature** .

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 **[M]0.15 % (by vol) formic acid** and spin to elute. Also add this eluate to the "eluate tube".

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: 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%.



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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 10m

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

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

38 10m

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

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 µl 20 µg/µl TMT reagent** i.e. **200 µg** 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** .



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44 10m

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

Note: It is a good practice to maintain the total volume to **100 µl** final reaction as it helps in reducing pipetting error when aliquoting **5 µl** 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: 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 [M]**5 % (by vol) final Hydroxyl amine solution** by dissolving in water from a [M]**50 % (by vol) stock solution** .

49 20m

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** .

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: This is important because if there is a sample loss during the downstream analysis or to further validate the findings.



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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: 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 1h 25m

Analyse each fraction by acquiring data in FT-FT-FT (MS3) HCD mode on a Orbitrap Fusion Lumos Mass spectrometer for **01:25:00** run for each fraction.

**C18 Stage-tip Protocol to desalt TMT labelled samples prior to MS analysis-Important step before final pooling of samples**

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.

Note: This is required to activate the C18 resin.

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)** .



**Citation:** Raja S. Nirujogi, Houjiang Zhou, Dario R Alessi (04/07/2021). Sample preparation protocol for total proteomic analysis of mouse tissues including brain. https://dx.doi.org/10.17504/protocols.io.bs3tngnn

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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 µl 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: Using new tubes is important to avoid contamination.

69 Add **30 µl 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



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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: 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: 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: 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: 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.



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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: 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: 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: 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: 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
.



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