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Sample preparation for TMT-based total and phospho-proteomic analysis of cells and tissues

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We use this protocol and it's working

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Abstract

Mass spectrometry-based proteomics and phosphoproteomics are highly sensitive and un-biased techniques to study the proteome and phosphoproteome at a global scale. Sample preparation is a key element for the generation of high quality, reproducible data. Here we provide a step-by-step protocol for processing material derived from cells or tissue samples. We recommend employing S-Trap assisted tryptic digestion followed by a TiO2-based phosphopeptide enrichment to achieve the highest possible reproducibility across experimental replicates. We also provide 10 or 16 plex Tandem Mass Tags (TMT) multiplexing strategy in combination with High-pH reversed-phase fractionation to achieve high coverage for phosphoproteomic analysis. The nano-liquid chromatography and High-resolution mass spectrometry instrument settings for both MS2 and Synchronous precursor selection MS3 data acquisition on Orbitrap Lumos Tribrid mass spectrometer are also described. Using these protocols, we routinely identify and quantify >35,000 phosphosites and ~10,000 protein groups.

Attachments

787-2015.docx

68KB

Materials

Materials

Consumables:

1. 1.5 ml protein low bind Eppendorf tubes (Eppendorf™ #022431081)
2. 2 ml protein low bind Eppendorf tubes (Eppendorf™ #0030108132)
3. Precellys Cryolys tissue homogenizer tubes (Precellys® Ceramic kit 2,8 mm, pre-filled with ceramic beads)
4. 15 ml falcon tubes
5. 15 ml racks
6. Marker pen
7. Pipette set (1 ml, 200 µl, 100 µl, 20 µl, 10 µl)
8. Pipette tips low binding (1 ml, 250 µl, 10 µl, Star labs Bevelled tips refill # S1111-3700, S1111-1706, S1111-6700)
9. PPE kit (Lab coat, gloves, safety glasses)
10. Dry ice
11. Liquid Nitrogen
12. Ice bucket
13. 1.5 ml Eppendorf tubes rack
14. 96 well plates - clear (Geneier Bio-one #655101)
15. 2 ml tubes (Axygen™ MCT200C)
16. 16-gauge needle (Sigma Aldrich # Z261378)
17. X100 20 ml amber glass EPA vial with cap and seal (Thermo Scientific™ EPA Screw Vial Assembled Kit. Fisher Scientific # 11543750)**
18. X72 40 ml amber glass EPA vial with cap and seal (Thermo Scientific™ EPA, TOC, and Scintillation Vials & Closures. Fisher Scientific # 12418656)**
19. Millipore pH Strips (VWR # 1.09584.0001)
20. CryoLys evolution homogenizer (Bertin technologies)
21. Hard tissue homogenizing CK28 – 2 ml (CAT. NO.: P000911-LYSK0-A)
22. S-Trap midi columns (https://www.protifi.com/)
23. Sep-Pak Vac 1cc (50 mg) tC18-Cartridges (Waters # WAT054960)
24. XBridge BEH C18 Column, 130A, 3.5 µm, 4.6 × 250 mm (Waters # 186003943)
25. 96 well 2.2 ml deep well plates (Fisher Scientific # 10089910)
26. Evotips (EvoSep #EV2013 EVOTIP PURE, 10×96 TIPS)
27. Acclaim PepMap 100 100 µm*cm nano viper trap column (Thermo Fisher Scientific # 164946)
28. Easy-Spray PepMap RSLC C18 2 µm, 50 cm x 75 µm (Fisher Scientific #16692027)

Note

** **Note**: Prepare all stock and working reagents in these amber vials to store either at room temperature or at 4 °C depending on the reagent (store as per the protocol).

Reagents:

SDS Lysis Buffer: Final 2% (by mass) SDS in

	A	B
	Triethylammonium bicarbonate pH 8.5*	100 mM
	sodium orthovanadate	1 mM
	NaF	50 mM
	b- glycerophosphate	10 mM
	sodium pyrophosphate	5 mM
	microcystin-LR	1 µg/ml
	complete EDTA-free protease inhibitor cocktail (Roche)	

*TEABC, this is the natural pH of this buffer and made from a 1 M TEABC stock purchased from Sigma Catalogue number# T7408-500 ml.

1. 20% (by mass) aqueous SDS stock
2. BCA protein assay kit (Pierce # 23225)
3. Tris (2-carboxyethyl) phosphine (TCEP) (Sigma Aldrich # 75259-10G).[a]
4. Iodoacetamide (Sigma # I1149)
5. LC-MS grade Trifluoroacetic acid (TFA) (Sigma# 302031-100 ML).[b]
6. S-Trap protein binding buffer (90% (by vol) aqueous LC grade methanol containing a final concentration of 100 mM TEABC made from a 1 M TEABC stock purchased from Sigma Aldrich # T7408-500 ML)
7. Sequencing grade trypsin (5 X 20 µg pack. Promega #V5111).[c]
8. TPCK treated Trypsin from bovine pancreas (Sigma Aldrich # T1426-100MG)
9. Methanol (VWR # 1.06035.2500)
10. LC-MS grade Acetonitrile (VWR # 1.00030.2500)
11. LC grade Formic acid (Sigma # 695076)
12. Sep-Pak Purification: Activation buffer (100% Acetonitrile (ACN) (by vol)
13. Sep-Pak Purification: Equilibration buffer (0.1% TFA (by vol) aqueous)
14. Sep-Pak Purification: Wash buffer (0.1% formic acid (by vol) aqueous)
15. Sep-Pak Purification: Elution buffer (0.1% formic acid (by vol) in 50% ACN (by vol) aqueous)
16. Empore C18 disks, 47 mm (CDS analytical #2215)[d]
17. High Select™ Phosphopeptide Enrichment Kits (Thermo Fisher Scientific #A32993)
18. TMTpro™ 16plex Label Reagent Set (Thermo Fisher Scientific # A44520)
19. Anhydrous Acetonitrile (Sigma Aldrich #271004)
20. 50% (by vol) Hydroxylamine by mass (Sigma Aldrich # 467804)
21. LC buffer (0.1% (by vol) Formic acid in 3% (by vol) Acetonitrile)

22. Solvent-A1 (0.1% (by vol) TFA)
23. Solvent-A2 (0.1% (by vol) Formic acid
24. Solvent-B1 (50% (by vol) acetonitrile in 0.1% (by vol) TFA)
25. Solvent-B2 (60% (by vol) acetonitrile in 0.1% (by vol) Formic acid)

Note

Notes:

[a] Prepare and store 10 µL aliquots of 1 M TCEP in Milli-Q H_2O. Prior to use dilute the [M] 1 Molarity (M) TCEP solution 10X in [M] 300 millimolar (mM) TEABC to generate a stock solution of [M] 0.1 Molarity (M) TCEP in [M] 300 millimolar (mM) TEABC.

[b] Prepare 20% vol/vol stock in an amber bottle and store at 4 °C for up to six months. TFA is toxic, must be prepared in fume hood using a suitable glassware.

[c] Store stocks in -20 °C freezer and thaw trypsin stock just before the digestion step.

[d] 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.

Equipment:

1. Pulveriser kit (https://cellcrusher.com/)
2. -80 °C deep freezer, -20 °C freezer and 4 °C fridge
3. Benchtop centrifuge (VWR)
4. Eppendorf centrifuge
5. Milli-Q water system
6. Orbital shaker
7. pH meter
8. Plate reader for Protein quantification (BioTek Epoch)
9. Diagenode Bioruptor plus sonication system
10. Eppendorf Thermomixer with ThermoTop, 0.5 ml, 1.5 ml, 2 ml and 7 ml tubes compatible heating blocks
11. Thermo Savant Speedvac system (#SPD140DDA)
12. 1.5 ml tube floaters
13. Branson water bath sonicator
14. Dionex RSLC 3000 nano-LC system
15. Dionex RSLC 3000 LC system for Offline fractionation with Auto sampler or Fraction collector, micro pump and VWD detector
16. Orbitrap Fusion Lumos Tribrid Mass spectrometer

17. Thermo Savant Speed vac system (#SPD140DDA)
18. Nanodrop 1000 (Thermo Fisher Scientific)
19. Rubber bulb # Fisher brand™ Rubber Pipette Bulb# 12446180

Protein LoBind tubes **Eppendorf** **Catalog #**022431081

96-Well Microplate Flat Bottom non-sterile Polystyrene Clear 10/Pack 100/Case **greiner bio-one** **Catalog #**655101

Stainless steel 316 syringe needle pipetting blunt 90° tip **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**Z261378

Thermo Scientific™ EPA Screw Vial Assembled Kit 20mL amber glass EPA vial with cap and seal **Thermo Fisher Scientific** **Catalog #**11543750

Thermo Scientific™ EPA TOC and Scintillation Vials & Closures **Thermo Fisher Scientific** **Catalog #**12418656

pH indicator strips mid range **VWR International (Avantor)** **Catalog #**1.09584.0001

Hard Tissue homogenizing CK28 **BERTIN CORP** **Catalog #**P000911-LYSK0-A

Sep-Pak tC18 1 cc Vac Cartridge 50 mg Sorbent per Cartridge **Waters** **Catalog #**WAT054960

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

BRAND™ Deep Well Plates **Fisher Scientific** **Catalog #**10680763

EV2013 EVOTIP PURE 10×96 TIPS **EVOSEP** **Catalog #**EV2013

EASY-Spray™ C18 LC Columns, 2µm particle size, 250mm Length x 75µm I.D. **Thermo Fisher** **Catalog #**ES802

Triethylammonium bicarbonate (TEAB) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T7408

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

Tris(2-carboxyethyl)phosphine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**75259

Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I1149-5G

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

Seq Grade Modified Trypsin, 100ug (5 × 20ug) **Promega** **Catalog #**V5111

TPCK-trypsin **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T1426-50MG

Methanol LiChrosolv® hypergrade for LC-MS Supelco® **VWR International (Avantor)** **Catalog #**1.06035.2500

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

LC-grade Formic acid **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**695076

Empore™ Extraction Disc Anion C18 **CDC** **Catalog #**2215

High-Select™ TiO2 Phosphopeptide Enrichment Kit **Thermo Fisher** **Catalog #**A32993

Thermo Scientific™ TMTpro™ 16plex Label Reagent Set **Thermo Fisher Scientific** **Catalog #**PIA44520

Acetonitrile **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**271004

Hydroxylamine solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**467804

Troubleshooting

Lysate preparation: For cells

25m

1 Prepare cells at a suitable confluency ~70 to 80% in a 15 cm dish. Ensure to have sufficient replicates, preferably 4 replicates per condition.

Note

Notes:
- The suitable starting material for an in-depth Phosphoproteomic analysis requires a minimum starting material of 3 mg protein amounts. If sufficient protein amounts not achievable from a single 15 cm dish, consider scaling up to pool from three 15 cm dishes per replicate in each condition.
- Phosphoproteomic sample preparation is lengthy and runs over a week period including several quality checks that need to be performed. It is possible that one or few samples may fail quality check, thus we recommend having a minimum of six replicates for each condition.

2 Wash cells with 5 mL plain DMEM medium and wash with 5 mL PBS.

Note

Note: All steps need to be performed with non-autoclaved low-binding pipette tips. This is to ensure not having any polymer contamination.

3 Add 700 µL of SDS lysis buffer to the dish and scrape it using a suitable scrapper, transfer the lysate into 1.5 ml low bind Eppendorf tube.

4 Boil samples at 95 °C for 00:05:00, cool them On ice and subject samples to sonication using Bioruptor, 30 sec/ON and 30 sec/OFF per cycle for a total of 15 cycles.

5m

Note

Note: If the protein lysate appears to be viscous, then consider using a probe sonicator.

5 Centrifuge samples at 20000 x g, 00:20:00 and transfer the supernatant to a new 1.5 ml low bind Eppendorf tubes.

20m

6 Take an aliquot for protein estimation using BCA assay kit.

Note

Note: For cells we recommend having 1:10 dilution and to have standards with six points e.g., 125 µg/µL , 250 µg/µL , 500 µg/µL , 750 µg/µL , 1000 µg/µL , 1500 µg/µL , and 2000 µg/µL BSA as standards.

7 Transfer lysates to -80 °C freezer until further analysis.

Lysate preparation: For tissue samples

2m

8 Measure the wet weight of the tissue sample and always maintains samples on dry ice.

9 Transfer tissue samples to 2 mL Precellys Cryoyls-vials and add 1 mL of SDS lysis buffer.

10 Place vials in Precellys homogenizer and use a program with 3 cycles (2000 rpm for 30 sec ON and 20 sec Pause per cycle).

11 Centrifuge samples at 2000 x g, 00:02:00 .

2m

Note

Note: Observe NO tissue chunks remain in the vial. If any, repeat homogenization for another 2 cycles.

12 Transfer samples to new 1.5 ml low bind Eppendorf tubes and follow the steps described from step 4 to step 7.

Sample preparation for S-Trap assisted digestion

18h 59m

13 Take 3 mg of protein for total and Phosphoproteomic analysis in a 2 ml low bind Eppendorf tubes.

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

15 Incubate on a Thermomixer for 00:30:00 at 60 °C temperature with a gentle agitation. 30m

16 Bring tubes to Room temperature and add a 1 in 10 dilution of freshly prepared [M] 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.

17 Incubate in dark on a Thermomixer at Room temperature for about 00:30:00 with a gentle agitation. 30m

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

19 Incubate on a Thermomixer for 00:20:00 at Room temperature with a gentle agitation. 20m

20 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).

21 Transfer lysates into a 15 ml falcon tube.

22 Add a final 1% (by vol) from a 20% (by vol) stock solution of Trifluoroacetic acid.

23 Dilute the samples to in 7 times the current volume of the mixture in of S-Trap wash buffer (90% (by vol) methanol in [M] 0.1 Molarity (M) TEABC pH 7.1 v/v) (for examples if sample volume is 50 µL, add 300 µL of S-Trap wash buffer (90% (by vol) methanol in [M] 0.1 Molarity (M) TEABC pH 7.1 (v/v)). Perform gentle vortex and transfer samples by pipetting up/down for few times to avoid any clumps.

Note

Note: We recommend processing a maximum of 24 samples at once. To avoid mistakes, number samples from 1 to 24 at every sub-sequent step.

24 Prepare an S-Trap midi column in a 15 ml falcon tube.

25 Add the diluted protein mixture to the column.

26 Centrifuge briefly to capture the protein particles at 2000 x g, Room temperature, 00:04:00 . 4m

Note

Note: It is possible that the sample may not flowthrough completely. In such cases increase the centrifugation speed in a step-wise manner but not exceeding > 4000 x g .

27 Wash column with 3.5 mL of S-Trap buffer a total of 4 times (spin 2000 x g, 00:04:00 between washes). 4m

Note

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

28 Move the S-Trap column to a clean 15 ml tube for digestion.

29 Add a 400 µL solution of freshly dissolved trypsin+Lys-C containing 30 µg for each sample freshly dissolved in [M] 100 millimolar (mM) TEABC*. Simultaneously add 400 µL of TPCK treated trypsin in [M] 100 millimolar (mM) TEABC containing 300 µg for each sample.

30 Centrifuge briefly at 200 x g, 00:01:00 . 1m

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

32 Cap the tubes and incubate at 47 °C without shaking for 01:30:00 on a Thermomixer with a 15 ml heating block. 1h 30m

Note

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

33 Incubate samples on Thermomixer for 16:00:00 at Room temperature . 16h

Note

Note: Do not shake.

34 Add 500 µL of [M] 50 millimolar (mM) TEABC then spin to elute and place the eluate in a new 15ml falcon tube termed “eluate tube”.

35 Next, add 500 µL of 0.15% (by vol) Formic Acid and spin to elute. Also add this eluate to the “eluate tube”.

36 Finally, add 500 µL of 80% (by vol) Acetonitrile in 0.15% (by vol) formic acid and spin to elute. Also add this eluate to the “eluate tube”. Repeat this step two more times.

Note

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

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

Note

Note: Analyse data with a 70 min 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%.

38 Vacuum dry the remaining peptide amount and store in -80 °C deep freezer until the Sep-Pak purification.

Sep-Pak purification

44m

39 Dissolve vacuum dried peptides by adding 1 mL of 1% TFA (by vol) aqueous and place the tubes on a Thermomixer at Room temperature for 00:30:00 shaking at 1800 rpm .

30m

40 Centrifuge tubes at high speed 17000 x g, Room temperature, 00:10:00 and place tubes aside for peptide purification using Sep-Pak cartridges.

10m

41 Place Sep-Pak Vac 1 cc (50 mg) tC18 cartridges each in 15 ml falcon tubes.

Note

Note: The capacity of the Sep-Pak is ~5 to 8%, e.g. 50 mg cartridge can be used with up to 2-3 mg of peptide digest. One column wash equals to 1 cc = 1 ml of buffer.

42 Add 1 mL of Activation buffer (100% ACN by vol).

43 Centrifuge at 50 x g, Room temperature, 00:01:00 . 1m

44 Repeat step 42 for a total of 4 column washes and discard the flowthrough.

45 Add 1 mL of equilibration buffer (0.1% TFA (by vol) aqueous).

46 Centrifuge at 50 x g, Room temperature, 00:01:00 . 1m

47 Repeat step 45 for a total of 4 column washes and discard the flowthrough.

48 Load acidified peptide digest slowly onto the column.

Note

Note: DO NOT CENTRIFUGE. Let the column drain on gravity. If required, push the sample to drain one/two drops using rubber bulb.

49 Reapply the collected flowthrough onto the column and save the flowthrough.

50 Add 1 mL of wash buffer (0.1% formic acid (by vol) aqueous).

51 Centrifuge at 50 x g, Room temperature, 00:01:00 . 1m

52 Repeat step 50 for a total of 4 column washes and discard the flowthrough.

53 Place columns onto 1.5 ml low bind Eppendorf tubes for elution.

Note

Note: Use 200 µl pipette tip to place in between column and Eppendorf tube surface at the top such that the column can be lifted, not touching the bottom of the tube.

54 Add 350 µL of elution buffer (0.1% formic acid (by vol) in 50% ACN (by vol) aqueous). Let the buffer elute peptides by gravity.

55 Repeat step 54 for two more times. After final elution discard columns, vortex tubes and centrifuge at 17000 x g, Room temperature, 00:01:00 . 1m

56 Take 5% by vol for total proteomic analysis.

57 A small aliquot ~0.1% can be taken for the verification of tryptic digestion. Submit these samples for mass spectrometry (MS) analysis.

58 Snap freeze samples on dry-ice and vacuum dry using Speed Vac concentrator and store samples in -80 °C freezer until Phosphopeptide enrichment.

Phosphopeptide enrichment using TiO_2 56m 30s

59 Label four sets of 2 ml low bind Eppendorf tubes.

60 Dissolve Sep-Pak purified peptide digest by adding 200 µL of binding buffer (provided with the kit). Place samples on a Thermomixer for 00:30:00 at Room temperature at 1800 rpm agitation. 30m

61 Centrifuge samples at 17000 x g, Room temperature, 00:05:00 and transfer supernatant to new 1.5 ml low bind Eppendorf tubes. 5m

Note

Note: DO NOT collect any precipitate that may block TiO_2 tips. Check peptide sample pH: pH should be < pH 3.0 .

62 Take High-select Phosphopeptide enrichment kit (Thermo Fisher Scientific).

Note

Note: Equilibrate all solutions of the kit to room temperature prior to enrichment experiment (00:30:00 at Room temperature). Securely tighten buffer bottle caps to prevent evaporation and store unused buffers and columns at 4 °C .

63 Label the TiO_2 spin tips with a marker.

Note

Note: We recommend following 1 to 24 (if you are processing 24 samples). Place centrifuge column adaptor (provided with the kit) in a 2 ml low bind Eppendorf tubes and insert TiO_2 spin tip into the adaptor.

64 Add 20 µL of Wash Buffer and centrifuge at 3000 x g, 00:02:00 . 2m

Note

Note: All centrifugation steps for this protocol needs to be done at Room temperature .

65 Add 20 µL of Binding/Equilibration Buffer and centrifuge at 3000 x g, 00:02:00 . 2m

66 Discard the flowthrough. Save the microcentrifuge tube for later "Wash column" step 1.

67 Transfer the equilibrated TiO_2 spin tips along with the centrifuge column adaptor into a new 2 ml low bind Eppendorf tubes.

68 Apply 200 µL of suspended peptide sample to the spin tip. Centrifuge at 1000 x g, 00:05:00 . 5m

69 Reapply sample in the microcentrifuge tube to the spin tip. Centrifuge at 1000 x g, 00:05:00 . 5m

Note

Note: If needed save the flowthrough for other PTM enrichment as Acetylation or Ubiquitinome analysis.

70 Transfer the TiO_2 spin tips along with the centrifuge column adaptor into a new 2 ml low bind Eppendorf tubes.

71 Wash column by adding 20 µL of Binding/Equilibration Buffer. Centrifuge at 3000 x g, 00:02:00 . 2m

72 Wash column by adding 20 µL of Wash Buffer. Centrifuge at 3000 x g, 00:02:00 . 2m

73 Repeat steps 71 and 72 in a sequential order.

74 Wash column by adding 20 µL of LC-MS grade water. Centrifuge at 3000 x g, 00:02:00 . 2m

75 Place TiO_2 spin tips into new 2 ml low bind Eppendorf tubes. Add 60 µL of elution buffer and centrifuge at 1000 x g, 00:01:00 . 1m

76 Repeat step 75 for a second round of elution. Discard spin tips, vortex samples and centrifuge at 17000 x g, 00:00:30 . 30s

77 Take 1% of the sample for Phosphopeptide enrichment verification by MS analysis.

78 Take 25 % of the sample as a back-up or for Data Independent Acquisition (DIA)-based MS analysis.

79 Snap freeze samples on dry ice and subject them for vacuum dryness using Speed Vac concentrator.

80 The Phosphopeptides needs to be purified prior to the Tandem mass tags (TMT) labelling using Sep-Pak purification protocol described in section **Sep-Pak purification**. Follow all steps except use 200 µL of elution buffer and repeat elution two more times for a total of 600 µL of eluates.

81 Snap freeze samples on dry ice and subject them for vacuum dryness using Speed Vac concentrator. Store samples in -80 °C freezer until the TMT labelling.

Tandem Mass Tags (TMT) labelling of peptides

3h 28m

82 Dissolve Sep-Pak purified total proteome and Phosphoproteomic samples by adding 30 µL of [M] 50 millimolar (mM) TEABC buffer. Place samples on a Thermomixer at Room temperature with an agitation at 1800 rpm for 00:20:00 . 20m

83 Take out TMT kit from -80 °C freezer and equilibrate it to reach Room temperature .

84 Dissolve 800 µg of each of the TMT mass tag reagents within the 10 or 16-plex TMT reagent kit with 80 µL of 100% by vol anhydrous acetonitrile to obtain 10 µg/µL concentration for each TMT reporter tag.

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.

85 Transfer dissolved peptides into a 0.5 ml low bind Eppendorf tubes.

86 Add 20 µL of 10 µg/µL TMT reagent i.e., 200 µg .

87 Give a gentle vortex and brief spin 2000 x g, 00:01:00 . 1m

88 Place samples on a Thermomixer and incubate at Room temperature for 02:00:00 with a gentle agitation 800 rpm . 2h

89 Add another 50 µL of [M] 50 millimolar (mM) TEABC 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 . 11m

Note

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.

90 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 Speed Vac.

Note

Note: It is important to verify the labelling efficiency of each TMT mass tag is and it should label > 98%, by analysing on Mass spec. We recommend doing this employing a 145 min 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 TMT-reporter 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. Alternatively, use in-house generated tool to normalise and adjust the volumes: https://samplepooler.proteo.info/).

91 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.

92 Thaw stored TMT labelled samples from step 91 to Room temperature .

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

94 Add 5 µL of 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

95 Pool all samples into a single tube.

96 Take 20% of the reaction i.e. 220 µL (For 16 plex-TMT experiment take 320 µL) 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.

97 Snap freeze the remaining 880 µL reaction and vacuum dry using Speed Vac.

98 Submit samples to MS facility for high pH fractionation.

99 Dissolve the digested peptide by adding 120 µL of High-pH Solvent-A ([M] 10 millimolar (mM) Ammonium formate pH 10.0). Place the sample on a Thermomixer with an agitation at 1800 rpm for 00:30:00 . Centrifuge at 17000 x g, 00:05:00 . 35m

100 Verify the pH to be ~ pH 10.0 . If pH appears to be low, adjust with Ammonium hydroxide (38% (by vol) by adding 1 µL and recheck the pH.

101 Ensure the LC-solvents are as Solvent-A ([M] 10 millimolar (mM) Ammonium formate pH 10.0); Solvent-B (90% ACN (v/v) in [M] 10 millimolar (mM) Ammonium formate pH 10.0).

Note

Note: Adjust the pH with 30% Ammonium Hydroxide.

102 Prepare the LC method by following the below gradient:

	A	B	C
	Time (min)	Nano pump Flow rate (µl/min)	% of Solvent-B
	0.0	0.275	3.0
	5.0	0.275	3.0
	20.0	0.100	3.0
	10.0	0.100	10.0
	50.0	0.100	40.0
	55.0	0.100	90.0
	62.0	0.100	90.0
	62.5	0.100	3.0
	70.0	0.100	3.0
	70.1	0.0100	3.0

103 Set the fraction collection time as Start time (min) 5.5 and End time (min) 62.0.

104 Collect a total of 96 fractions by keeping the fraction collection for 00:01:00 for each fraction. 1m

105 Concatenate by pooling distant fractions e.g. A1+D1, A2+D2, B1+E1, B2+E2 and so on to a total of 48 fractions in a 1.5 ml low bind Eppendorf tubes for LC-MS/MS analysis.

106 Snap freeze and vacuum dry using Speed Vac concentrator.

107 Prepare 2 µg of each fraction in 15 µL in LC buffer (0.1% (by vol) formic acid in 3% (by vol) Acetonitrile) and submit each fraction to the mass spectrometry facility.

108 Analyse each fraction by acquiring data in FT-FT-FT (MS3) HCD mode on a Orbitrap Fusion Lumos Mass spectrometer for 85 min run for each fraction.

LC-MS/MS analysis on Orbitrap Lumos Tribrid mass spectrometer for Phosphoproteomic analysis

109 Take 2 µg of each fraction from Phosphoproteomic experiment, transfer into LC vial and place it in LC autosampler tray.

110 Construct LC and MS method using the below settings.

111 **LC Method**: Dionex RSLC 3000 Ultimate LC system, 2 cm trap column and 50 cm analytical column connected and interfaced with Easy nano-source (Thermo Fisher Scientific).

	A	B	C	D
	No	Time (min)	Nano pump Flow rate (µl/min)	% Solvent-B
	1	0	0.3	3
	2	5	0.3	8
	3	75	0.3	25
	4	85	0.3	35
	5	85.5	0.3	95
	6	93	0.3	95
	7	93.5	0.3	3
	8	100	0.3	3
	9	100	Stop	

112 **Mass spectrometer parameters**: Refer below settings to construct FT-FT-HCD (MS2) method:

	A	B
	Method Summary	
	Method Settings	
	Application Mode	Peptide
	Method Duration (min)	100
	Global Parameters	
	Ion Source	
	Use Ion Source Settings from Tune	True
	FAIMS Mode	Not Installed
	MS Global Settings	
	Infusion Mode	Liquid Chromatography
	Expected LC Peak Width (s)	30
	Advanced Peak Determination	True
	Default Charge State	2
	Internal Mass Calibration	Off
	Experiment#1 [MS]	
	Start Time (min)	0
	End Time (min)	100
	Master Scan	
	MS OT	
	Detector Type	Orbitrap
	Orbitrap Resolution	120000
	Mass Range	Normal
	Use Quadrupole Isolation	True
	Scan Range (m/z)	375-1400
	RF Lens (%)	32
	AGC Target	Standard

	A	B
	Maximum Injection Time Mode	Custom
	Maximum Injection Time (ms)	50
	Micro scans	1
	Data Type	Profile
	Polarity	Positive
	Source Fragmentation	Disabled
	Scan Description	
	Filters	
	MIPS	
	Monoisotopic Peak Determination	Peptide
	Charge State	
	Include charge state(s)	2-7
	Include undetermined charge states	False
	Dynamic Exclusion	
	Use Common Settings	False
	Exclude after n times	1
	Exclusion duration (s)	45
	Mass Tolerance	ppm
	Low	10
	High	10
	Exclude Isotopes	True
	Perform dependent scan on single charge state per precursor only	True
	Intensity	
	Filter Type	Intensity Threshold
	Intensity Threshold	5.00E+04
	Precursor Fit	
	Fit Threshold (%)	70

	A	B
	Fit Window (m/z)	0.7
	Data Dependent	
	Data Dependent Mode	Number of Scans
	Number of Dependent Scans	15
	Scan Event Type 1	
	Scan	
	ddMS² OT HCD	
	Isolation Mode	Quadrupole
	Isolation Window (m/z)	0.7
	Isolation Offset	Off
	Activation Type	HCD
	Collision Energy Mode	Fixed
	HCD Collision Energy (%)	30
	Detector Type	Orbitrap
	Orbitrap Resolution	50000
	Mass Range	Normal
	Scan Range Mode	Define First Mass
	First Mass (m/z)	110
	AGC Target	Custom
	Normalized AGC Target (%)	200
	Maximum Injection Time Mode	Custom
	Maximum Injection Time (ms)	120
	Micro scans	1
	Data Type	Profile
	Use EASY-IC™	False
	Scan Description	

113 Export the MS raw data for database searches using MaxQuant or MS-Fragger. Analyse database search results using Perseus software package or R or MS-Stats or Python for statistical analysis.

LC-MS/MS analysis on Orbitrap Lumos Tribrid mass spectrometer for total proteomic analysis

114 Take 2 µg of each fraction from Phosphoproteomics experiment, transfer into LC vial and place it in LC autosampler tray.

115 Construct LC and MS method using the below settings.

116 **LC Method**: Dionex RSLC 3000 Ultimate LC system, 2 cm trap column and 50 cm analytical column connected and interfaced with Easy nano-source (Thermo Fisher Scientific).

	A	B	C	D
	No	**Time** (min)	**Nano pump Flow rate** (µl/min)	**% Solvent-B**
	1	0	0.3	3
	2	5	0.3	8
	3	7	0.3	25
	4	85	0.3	35
	5	86	0.3	95
	6	92	0.3	95
	7	93	0.3	3
	8	100	0.3	3
	9	100	Stop	

117 **Mass spectrometer parameters**: Refer below settings to construct FT-IT-HCD-FT-HCD (MS3) method:

	A	B
	Method Summary	
	Method Settings	

	A	B
	Application Mode	Peptide
	Method Duration (min)	100
	Global Parameters	
	Ion Source	
	Use Ion Source Settings from Tune	True
	FAIMS Mode	Not Installed
	MS Global Settings	
	Infusion Mode	Liquid Chromatography
	Expected LC Peak Width (s)	30
	Advanced Peak Determination	True
	Default Charge State	2
	Internal Mass Calibration	Off
	Experiment#1 [MS]	
	Start Time (min)	0
	End Time (min)	100
	Cycle Time (sec)	2
	Master Scan	
	MS OT	
	Detector Type	Orbitrap
	Orbitrap Resolution	120000
	Mass Range	Normal
	Use Quadrupole Isolation	True
	Scan Range (m/z)	350-1500
	RF Lens (%)	30
	AGC Target	Standard
	Maximum Injection Time Mode	Custom
	Maximum Injection Time (ms)	50

	A	B
	Micro scans	1
	Data Type	Profile
	Polarity	Positive
	Source Fragmentation	Disabled
	Scan Description	
	Filters	
	MIPS	
	Monoisotopic Peak Determination	Peptide
	Charge State	
	Include charge state(s)	2-7
	Include undetermined charge states	False
	Dynamic Exclusion	
	Use Common Settings	False
	Exclude after n times	1
	Exclusion duration (s)	45
	Mass Tolerance	ppm
	Low	10
	High	10
	Exclude Isotopes	True
	Perform dependent scan on single charge state per precursor only	True
	Intensity	
	Filter Type	Intensity Threshold
	Intensity Threshold	5.00E+03
	Precursor Fit	
	Fit Threshold (%)	70
	Fit Window (m/z)	0.7
	Data Dependent	

	A	B
	Data Dependent Mode	Cycle Time
	Time between Master Scans (sec)	2
	Scan Event Type 1	
	Scan	
	ddMS² IT HCD	
	Isolation Mode	Quadrupole
	Isolation Window (m/z)	0.7
	Isolation Offset	Off
	Activation Type	HCD
	Collision Energy Mode	Fixed
	HCD Collision Energy (%)	32
	Detector Type	Ion Trap
	Ion Trap Scan Rate	Rapid
	Mass Range	Normal
	Scan Range Mode	Define m/z range
	Scan Range (m/z)	200-1400
	AGC Target	Custom
	Normalized AGC Target (%)	200
	Maximum Injection Time Mode	Custom
	Maximum Injection Time (ms)	50
	Micro scans	1
	Data Type	Centroid
	Scan Description	
	Filters	
	Precursor Selection Range	
	Selection Range Mode	Mass Range
	Mass Range (m/z)	400-1400

	A	B
	Precursor Ion Exclusion	
	Exclusion mass width	ppm
	Low	25
	High	25
	Isobaric Tag Loss Exclusion	
	Reagent	TMTpro
	Data Dependent	
	Data Dependent Mode	Scans Per Outcome
	Scan Event Type 1	
	Scan	
	ddMS^3 OT HCD	
	MS^n Level	3
	Synchronous Precursor Selection	True
	Number of SPS Precursors	10
	MS Isolation Window (m/z)	0.7
	MS2 Isolation Window (m/z)	2
	Isolation Offset	Off
	Activation Type	HCD
	HCD Collision Energy (%)	55
	Detector Type	Orbitrap
	Orbitrap Resolution	50000
	Mass Range	Normal
	Scan Range Mode	Define m/z range
	Scan Range (m/z)	110-500
	AGC Target	Standard
	Maximum Injection Time Mode	Custom
	Maximum Injection Time (ms)	120

	A	B
	Micro scans	1
	Data Type	Profile
	Use EASY-IC™	False
	Scan Description	
	Number of Dependent Scans	10

118 Export the MS raw data for database searches using MaxQuant or MS-Fragger. Analyse database search results using Perseus software package or R or MS-Stats or Python for statistical analysis.