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

**Authors: Ilham Seffouh1, Tran Le Cong Huyen Bao Phan1, Toan K. Phung1, Dario R Alessi1 and Raja S. Nirujogi1**

**1 Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK**

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 11 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.

1. **Materials**
   1. **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 plate- clear (Geneier Bio-one #655101)
      15. 2 ml tubes (Axygen™ MCT200C)
      16. 16-gauge needle (# Z261378. Sigma Aldrich)
      17. X100 20 mL Amber Glass EPA Vial (Thermo Scientific™ EPA Screw Vial Assembled Kit, 20mL amber glass EPA vial with cap and seal. Fisher Scientific # 11543750)
      18. X72 40 mL Amber class EPA vial W Cap and seal (Thermo Scientific™ EPA, TOC, and Scintillation Vials & Closures. Fisher Scientific # 12418656)

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

* + 1. Millipore pH Strips (VWR # 1.09584.0001)
    2. CryoLys evolution homogenizer (Bertin technologies)
    3. Hard tissue homogenizing CK28 – 2 mL (CAT. NO.: P000911-LYSK0-A)
    4. S-Trap midi columns ([https://www.protifi.com/](https://www.protifi.com/)))
    5. Sep-Pak Vac 1cc (50 mg) tC18-Cartridges (Waters # WAT054960)
    6. XBridge BEH C18 Column, 130A, 3.5 um, 4.6 x 250 mm (Waters # 186003943)
    7. 96 well 2 ml deep well plates (Fisher Scientific # 10680763)
    8. Evotips (EvoSep #EV2013 EVOTIP PURE, 10×96 TIPS)
    9. Acclaim PepMap 100 100 μm\*cm nano viper trap column (Thermo Fisher Scientific # 11312263)
    10. Easy-Spray PepMap RSLC C18 2 μm, 50 cm x 75 um (Thermo Fisher Scientific #ES802)
  1. **Reagents:**
     1. SDS Lysis Buffer: Final 2% (by mass) SDS in 100 mM Triethylammonium bicarbonate pH 8.5 (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 mM sodium orthovanadate, 50 mM NaF, 10 mM β- glycerophosphate, 5 mM sodium pyrophosphate, 1 μg/ml microcystin-LR, and complete EDTA-free protease inhibitor cocktail (Roche)
     2. 20% (by mass) aqueous SDS stock
     3. BCA protein assay kit (Pierce # 23225)
     4. Tris (2-carboxyethyl) phosphine (TCEP) (Sigma Aldrich # 75259-10G). (Note: Prepare and store 10 µl aliquots of 1 M TCEP in Milli-Q H2O. Prior to use dilute the 1 M TCEP solution 10X in 300 mM TEABC to generate a stock solution of 0.1 M TCEP in 300 mM TEABC).
     5. Iodoacetamide (Sigma # I1149)
     6. LC-MS grade Trifluoroacetic acid (TFA) (Sigma# 302031-100ML). (Note: Prepare 20% vol/vol stock in an amber bottle and store at 4oC for up to six months. TFA is toxic must be prepared in fume hood using a suitable glassware)
     7. S-Trap protein binding buffer (90% (byvol) 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)
     8. Sequencing grade trypsin (5 X 20 μg pack. Promega #V5111). (Note: Store stocks in -20oC freezer and thaw trypsin stock just before the digestion step)
     9. TPCK treated Trypsin from bovine pancreas (Sigma Aldrich # T1426-100MG)
     10. Methanol (VWR # 1.06035.2500)
     11. LC-MS grade Acetonitrile (VWR # 1.00030.2500)
     12. LC grade Formic acid (Sigma # 695076)
     13. Sep-Pak Purification: Activation buffer (100% Acetonitrile (ACN) (by/vol)
     14. Sep-Pak Purification: Equilibration buffer (0.1% TFA (by/vol) aqueous)
     15. Sep-Pak Purification: Wash buffer (0.1% formic acid (by/vol) aqueous)
     16. Sep-Pak Purification: Elution buffer (0.1% formic acid (by/vol) in 50% ACN (by/vol) aqueous)
     17. Empore C18 disks, 47 mm (CDS analytical #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).
     18. High Select™ Phosphopeptide Enrichment Kits (Thermo Fisher Scientific #A32993)
     19. TMTpro™ 16plex Label Reagent Set (Thermo Fisher Scientific # A44520)
     20. Anhydrous Acetonitrile (Sigma Aldrich #271004)
     21. 50% (by vol) Hydroxylamine by mass (Sigma Aldrich # 467804)
     22. LC buffer (0.1% (by vol) Formic acid in 3% (by vol) Acetonitrile)
     23. Solvent-A1 (0.1% (by vol) TFA)
     24. Solvent-A2 (0.1% (by vol) Formic acid
     25. Solvent-B1 (50% (by vol) acetonitrile 0.1% (by vol) TFA)
     26. Solvent-B2 (60% (by vol) acetonitrile 0.1% (by vol) Formic acid)

**1.3) Equipment:**

* + 1. Pulveriser kit (<https://cellcrusher.com/>)
    2. -80oC deep freezer, -20oC freezer and 4oC 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.

1. **Protocol:**
   1. **Lysate preparation:**
      1. For cells:

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: 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). (Note: Phosphoproteomic sample preparation is lengthy and runs over a week period including several quality checks needs to be performed and it is possible one or few samples may fail quality check thus we recommend having a minimum of six replicates for each condition)

* + 1. Wash cells with 5 ml ice-cold plain DMEM medium and wash with 5ml ice-cold PBS (Note: All steps need to be performed with non-autoclaved lo-binding pipette tips. This is to ensure not having any polymer contamination)
    2. Add 700 µl of SDS lysis buffer to the dish and scrape it using a suitable scrapper, transfer the lysate into 1.5 ml lo-bind Eppendorf tube
    3. Boil samples at 95oC for five minutes, 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 (Note: If the protein lysate appears to be viscous, then consider using probe sonicator.)
    4. Centrifuge samples at 20,000 g for 20 minutes and transfer the supernatant to a new 1.5 ml lobind Eppendorf tubes
    5. Take an aliquot for Protein estimation using BCA assay kit (Note: For cells we recommend having 1:10 dilution and to have standards with six points e.g., 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1.0 mg/ml, 1.5 mg/ml, and 2.0 mg/ml BSA as standards)
    6. Transfer lysates to -80 freezer until further analysis
    7. For tissue samples:

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

* + 1. Transfer tissue samples to 2 ml Precellys Cryoyls-vials and add 1 ml of SDS lysis buffer.
    2. Place vials in Precellys homogenizer and use a program with 3 cycles (2000 rpm for 30 sec ON and 20 sec Pause per cycle)
    3. Centrifuge samples at 2,000 g for 2 min (Note: Observe NO tissue chunks remain in the vial. If any repeat homogenization for another 2 cycles)
    4. Transfer samples to new 1.5 ml low bind Eppendorf tubes and follow the steps described from 2.1.4 to 2.1.7)
  1. **Sample preparation for S-Trap assisted digestion:**
     1. Take 3 mg of protein for total and Phosphoproteomic analysis in a 2 ml lobind Eppendorf tubes
     2. Perform reduction by adding a 1 in 10 dilution of a solution of 0.1 M TCEP dissolved in 300 mM TEABC to bring final concentration of TCEP to 10 mM
     3. Incubate on a Thermomixer for 30 min at 60oC temperature with a gentle agitation.
     4. Bring tubes to room temperature and add a 1 in 10 dilution of freshly prepared 0.4 M iodoacetamide dissolved in water (Note it is critical that the samples are at room temperature prior to addition of iodoacetamide)
     5. Incubate in dark on a Thermomixer at room temperature for about 30 min with a gentle agitation.
     6. Quench alkylation by addition of a 1 in 10 dilution of 0.1 M TCEP dissolved in 300 mM TEABC to bring final concentration of TCEP to 10 mM.
     7. Incubate on a Thermomixer for 20 min at room temperature with a gentle agitation.
     8. Add SDS to a final concentration of 5% (by mass) from 20% (by mass) SDS stock.

(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).

* + 1. Transfer lysates into a 15 ml falcon tube.
    2. Add a final 1% (by vol) from a 20% (by vol) stock solution of Trifluoroacetic acid
    3. Dilute the samples to in 7 times the current volume of the mixture in of S-Trap wash buffer (90% (by vol) methanol in 0.1 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 0.1 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: We recommend processing a maximum of 24 samples at once and to avoid mistakes number samples from 1 to 24 at every sub-sequent step)
    4. Prepare an S-Trap midi column in a 15 ml falcon tube.
    5. Add the diluted protein mixture to the column.
    6. Centrifuge briefly to capture the protein particles at 2,000 g for 4 minutes at room temperature. (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 >4,000 g).
    7. Wash column with 3.5 ml of S-Trap buffer a total of 4 times (spin 2,000 g for 4 min between washes) (Note that the protein remains bound on the column and SDS and buffer components that affect trypsin digestion are removed).
    8. Move the S-Trap column to a clean 15 ml tube for digestion.
    9. Add a 400 μl solution of freshly dissolved trypsin+Lys-C containing 30 μg for each sample freshly dissolved in 100 mM TEABC (1:100): (Note we use 6.5μg of trypsin and 1 μg of Lys-C per 100 μg of protein). Simultaneously add 400 μl of TPCK treated trypsin in 100 mM TEABC containing 300 μg for each sample.
    10. Centrifuge briefly at 200 g for 1 min
    11. Collect flowthrough and reapply the trypsin solution back onto the column, being careful to avoid air bubbles
    12. Cap the tubes and incubate at 47oC without shaking for 1.5 hour on a Thermomixer with a 15 ml heating block (Note do not shake as this causes bubbles and damage the column)
    13. Incubate samples on Thermomixer for 16 hr at room temperature (Note do not shake)
    14. Add 500 µl of 50 mM TEABC then spin to elute and place the eluate in a new 15ml falcon tube termed “eluate tube”.
    15. Next, add 500 µl of 0.15% (by vol) Formic Acid and spin to elute. Also add this eluate to the “eluate tube”
    16. Finally, add 500 µl of 50% (by vol) Acetonitrile in 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)
    17. Take 1-2 µl of the combined eluate, vacuum dry and inject on MS to verify the digestion efficiency (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%).
    18. Vacuum dry the remaining peptide amount and store in -80oC deep freezer until the Sep-Pak purification.
  1. **Sep-Pak purification**:
     1. 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 30 min shaking at 1800 rpm
     2. Centrifuge tubes at high speed (17,000 g) at room temperature for 10 minutes and place tubes aside for peptide purification using Sep-Pak cartridges
     3. Place Sep-Pak Vac 1 cc (50 mg) tC18 cartridges each in 15 ml falcon tubes (Note: The capacity of the Sep-Pak is ~5 to 8%, e.g. 50 mg cartridge can be used with up to 2 to 3 mg of peptide digest. One column wash equals to 1 cc = 1 ml of buffer)
     4. Add 1 ml of Activation buffer (100% ACN by vol)
     5. Centrifuge at 50 g at room temperature for 1 min.
     6. Repeat step 2.2.4 for a total of 4 column washes and discard the flowthrough
     7. Add 1 ml of equilibration buffer (0.1% TFA (by vol) aqueous)
     8. Centrifuge at 50 g at room temperature for 1 min
     9. Repeat step 2.2.7 for a total of 4 column washes and discard the flowthrough
     10. Load acidified peptide digest slowly onto the column (Note: DO NOT CENTRIFUGE. Let the column drain on gravity if required push the sample to drain one/two drops using rubber bulb)
     11. Reapply the collected flowthrough onto the column and save the flowthrough
     12. Add 1ml of wash buffer (0.1% formic acid (by vol) aqueous)
     13. Centrifuge at 50 g at room temperature for 1 min.
     14. Repeat step 2.2.12 for a total of 4 column washes and discard the flowthrough
     15. Place columns onto 1.5 ml low bind Eppendorf tubes for elution (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)
     16. 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.
     17. Repeat step 2.2.16 for two more times. After final elution discard columns, vortex tubes and centrifuge at 17,000 g for one minute at room temperature
     18. Take 5% by vol for total proteomic analysis.
     19. A small aliquot ~0.1% can be taken for the verification of tryptic digestion. Submit these samples for mass spectrometry (MS) analysis.
     20. Snap freeze samples on dry-ice and vacuum dry using Speed Vac concentrator and store samples in -80 freezer until Phosphopeptide enrichment.
  2. **Phosphopeptide enrichment using TiO2:**
     1. Label four sets of 2 ml low bind Eppendorf tubes.
     2. Dissolve Sep-Pak purified peptide digest by adding 200 µl of binding buffer (provided with the kit). Place samples on a Thermomixer for 30 min at room temperature at 1800 rpm agitation.
     3. Centrifuge samples at 17,000 g for 5 min at room temperature and transfer supernatant to new 1.5 ml low bind Eppendorf tubes (Note: DO NOT collect any precipitate that may block TiO2 tips. Check peptide sample pH: pH should be < 3.0 pH).
     4. Take High-select Phosphopeptide enrichment kit (Thermo Fisher Scientific). (Note: Equilibrate all solutions of the kit to room temperature prior to enrichment experiment (30 min at room temperature). Securely tighten buffer bottle caps to prevent evaporation and store unused buffers and columns at 4°C.)
     5. Label the TiO2 spin tips with a marker (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 TiO2 spin tip into the adaptor.
     6. Add 20 μl of Wash Buffer and centrifuge at 3,000 g for 2 min. (Note: All centrifugation steps for this protocol needs to be done at room temperature)
     7. Add 20 μl of Binding/Equilibration Buffer and centrifuge at 3,000 g for 2 min.
     8. Discard the flowthrough. Save the microcentrifuge tube for later "Wash column" step 1.
     9. Transfer the equilibrated TiO2 spin tips along with the centrifuge column adaptor into a new 2 ml lobind Eppendorf tubes.
     10. Apply 200 μl of suspended peptide sample to the spin tip. Centrifuge at 1,000 g for 5 min.
     11. Reapply sample in the microcentrifuge tube to the spin tip. Centrifuge at 1,000 g for 5 min. (Note: If needed save the flowthrough for other PTM enrichment as Acetylation or Ubiquitinome analysis)
     12. Transfer the TiO2 spin tips along with the centrifuge column adaptor into a new 2 ml low bind Eppendorf tubes.
     13. Wash column by adding 20 μl of Binding/Equilibration Buffer. Centrifuge at 3,000 g for 2 min.
     14. Wash column by adding 20 μl of Wash Buffer. Centrifuge at 3,000 g for 2 min.
     15. Repeat steps 2.3.13 and 2.3.14 in a sequential order.
     16. Wash column by adding 20 μl of LC-MS grade water. Centrifuge at 3,000g for 2 min.
     17. Place TiO2 spin tips into new 2 ml low bind Eppendorf tubes. Add 60 μl of elution buffer and centrifuge at 1,000 g for 1 min.
     18. Repeat step 2.3.17 for a second round of elution. Discard spin tips, vortex samples and centrifuge at 17,000 g for 30 sec.
     19. Take 1% of the sample for Phosphopeptide enrichment verification by MS analysis.
     20. Take 25 % of the sample as a back-up or for Data Independent Acquisition (DIA)-based MS analysis.
     21. Snap freeze samples on dry ice and subject them for vacuum dryness using Speed Vac concentrator.
     22. The Phosphopeptides needs to be purified prior to the Tandem mass tags (TMT) labelling using Sep-Pak purification protocol described in section 2.2. Follow all steps except use 200 µl of elution buffer and repeat elution two more times for a total of 600 µl of eluates.
     23. Snap freeze samples on dry ice and subject them for vacuum dryness using Speed Vac concentrator. Store samples in -80 freezer until the TMT labelling.
  3. **Tandem Mass Tags (TMT) labelling of peptides:**
     1. Dissolve Sep-Pak purified total proteome and Phosphoproteomic samples by adding 30 µl of 50 mM TEABC buffer. Place samples on a Thermomixer at room temperature with an agitation at 1800 rpm for 20 min.
     2. Take out TMT kit from -80 freezer and equilibrate it to reach room temperature.
     3. 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: Dissolved TMT reagents are prone to hydrolysis so immediately after aliquoting store remainder reagent in -80oC deep freezer for long-term storage up to six months and try to avoid multiple freeze thaw cycles).
     4. Transfer dissolved peptides into a 0.5 ml low bind Eppendorf tubes.
     5. Add 20 µl of 10 µg/µl TMT reagent i.e., 100 µg.
     6. Give a gentle vortex and brief spin 2,000 g for 1 min.
     7. Place samples on a Thermomixer and incubate at room temperature for 2 hours with a gentle agitation, 800 rpm.
     8. Add another 50 μl of 50 mM TEAB buffer to make a final 100 μl reaction. Vortex, brief spin at 2,000 g for 1 min and incubate on a Thermomixer for 10 min.

(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).

* + 1. 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: 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/).
    2. Place remaining 95 µl of the reaction in -80 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.
    3. Thaw stored TMT labelled samples from step 2.4.10 to room temperature.
    4. Prepare 5% (by vol) final Hydroxyl amine solution by dissolving in water from a 50% (by vol) stock solution.
    5. 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 20 min.
    6. Pool all samples into a single tube.
    7. 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 this is important because if there is a sample loss during the downstream analysis or to further validate).
    8. Snap freeze the remaining 880 µl reaction and vacuum dry using Speed Vac.
    9. Submit samples to MS facility for high pH fractionation.
    10. Dissolve the peptide digest by adding 120 µl of High-pH Solvent-A (10 mM Ammonium formate pH 10.0). Place the sample on a Thermomixer with an agitation at 1,800 rpm for 30 min. Centrifuge at 17,000 g for 5min.
    11. Verify the pH to be ~ 10.0. If pH appears to be low adjust with Ammonium hydroxide (38% (by/vol) by adding 1 µl and recheck the pH.
    12. Ensure the LC-solvent are as Solvent-A (10 mM Ammonium formate pH 10.0); Solvent-B (90% ACN (v/v) in 10mM Ammonium formate pH 10.0). (Note: Adjust the pH with 30% Ammonium Hydroxide).
    13. Prepare the LC method by following the below gradient:

|  |  |  |
| --- | --- | --- |
| Time (minutes) | 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 |

* + 1. Set the fraction collection time as Start time (min) 5.5 and End time (min) 62.0.
    2. Collect a total of 96 fractions by keeping the fraction collection for 60 seconds for each fraction.
    3. 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 in a 1.5 ml low bind Eppendorf tubes for LC-MS/MS analysis.
    4. Snap freeze and vacuum dry using Speed Vac concentrator.
    5. 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.
    6. 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.
  1. **LC-MS/MS analysis on Orbitrap Lumos Tribrid mass spectrometer for Phosphoproteomic analysis**
     1. Take 2 µg of each fraction from Phosphoproteomic experiment, transfer into LC vial and place it in LC autosampler tray.
     2. Construct LC and MS method using the below settings.
     3. 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).

|  |  |  |  |
| --- | --- | --- | --- |
| No | Time (min) | Nano pump Flow rate (ul/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 | |

* + 1. Mass spectrometer parameters: Refer below settings to construct FT-FT-HCD (MS2) method:

|  |  |
| --- | --- |
| 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 |
| 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 |
| 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 |  |

* + 1. 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.
  1. **LC-MS/MS analysis on Orbitrap Lumos Tribrid mass spectrometer for total proteomic analysis**
     1. Take 2 µg of each fraction from Phosphoproteomics experiment, transfer into LC vial and place it in LC autosampler tray.
     2. Construct LC and MS method using the below settings.
     3. 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)

|  |  |  |  |
| --- | --- | --- | --- |
| 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 | |

* + 1. Mass spectrometer parameters: Refer below settings to construct FT-IT-HCD-FT-HCD (MS3) method:

|  |  |
| --- | --- |
| 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 |
| 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 |
| 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 |  |
| 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 |
| 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 |  |
| ddMS³ OT HCD |  |
| MSⁿ 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 |
| Micro scans | 1 |
| Data Type | Profile |
| Use EASY-IC™ | False |
| Scan Description |  |
| Number of Dependent Scans | 10 |

* + 1. 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.