**GFP Immunoprecipitation and Sample Preparation for Tandem Mass Tag (TMT) Mass Spectrometry Analysis**

Prosenjit Pal1,2, Raja S. Nirujogi1,2, Francesca Tonelli1,2, Dario Alessi1,2

1 MRC Protein Phosphorylation and Ubiquitylation Unit, University of Dundee, DD1 5EH, UK

2 Aligning Science Across Parkinson’s (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815

**Abstract:**

We describe a method to identify potential interactors of any Green Fluorescent Protein (GFP) tagged protein expressed in mammalian cells by GFP immunoprecipitation coupled to Tandem Mass Tag (TMT) mass spectrometry analysis. As an example, we used a GFP-tagged phosphoRab interactor protein (RILPL1-GFP), and its non-binding mutant (RILPL1 [R293A]-GFP, which cannot interact with phosphorylated Rab proteins) as a control.

Protocol overview:

**1) Transient transfection of HEK293 cells for expression of GFP-tagged proteins.**

**2) Preparation and quantification of cell lysates from HEK293 cells.**

**3) Immunoprecipitation of GFP-tagged proteins from cell lysates.**

**4) On-bead tryptic digestion and TMT labelling of immunoprecipitated proteins for LC-MS/MS mass spectrometry analysis.**

**Materials:**

1. **Reagents**
2. **For cell culture, transient transfection and GFP immunoprecipitation:**
* HEK293 cells (ATCC #CRL-1573) cultured in complete growth medium.
* Growth medium: Dulbecco’s Modified Eagle’s Medium (DMEM), High Glucose, no glutamine (GibcoTM, catalog number: 11960044, or equivalent) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Sigma #F7524, or equivalent), 2 mM L-glutamine (GibcoTM, catalog number: 25030024, or equivalent), Penicillin-Streptomycin 100U/mL (GibcoTM, catalog number: 15140122, or equivalent).
* 0.05% trypsin-EDTA (GibcoTM, catalog number: 25300054, or equivalent)
* Dulbecco's phosphate-buffered saline (PBS) (GIBCO. REF# 14190169)
* Linear polyethylenimine (PEI Max 40K. Polyscience #24765); 1 mg/ml (w/v) stock in de-ionised H2O, pH 7.4; sterile filtered.
* Transfection media (for HEK293FT cells): Opti-MEM Reduced Serum Medium (ThermoFisher Scientific #31985062)
* Plasmids for mammalian expression (pCMV vector):

FLAG-LRRK2 Y1699C (DU26486, available at MRCPPU Reagents and Services https://mrcppureagents.dundee.ac.uk)

RILPL1-GFP WT (DU27305, available at MRCPPU Reagents and Services https://mrcppureagents.dundee.ac.uk)

RILPL1-GFP R293A (DU68072, available at MRCPPU Reagents and Services https://mrcppureagents.dundee.ac.uk)

HA-Rab8A Q67L (DU51181, available at MRCPPU Reagents and Services https://mrcppureagents.dundee.ac.uk)

* Bradford Protein Assay Kit
* ChromoTek GFP-Trap Agarose Beads (Cat# gta-20)
* Lysis Buffer: 50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 150 mM NaCl, 0.5 mM EDTA, 1% (v/v) NP-40 Alternative (Merck #492016), 1X phosSTOP phosphatase inhibitor cocktail (PhosSTOP tablet: Roche, REF# 04906837001; to be added just before use) and 1X protease inhibitor cocktail (cOmplete EDTA-free protease inhibitor cocktail tablet: Roche, REF# 11873580001; to be added just before use)
* IP Wash Buffer:50 mM Tris-HCl pH 7.5, 150 mM NaCl
1. **For TMT mass spectrometry analysis:**
* Dithiothreitol (DTT) (Sigma Aldrich; Cat# D0632-10G) – Prepare fresh as a 100 mM Stock in Milli-Q H2O
* Iodoacetamide (IAA) (Sigma - I1149) – Prepare fresh as a 200 mM stock in Milli-Q H2O
* Urea (Thermo Scientific, Cat# 29700)
* Elution buffer I (to be made just before use): 2 M Urea, 50 mM Tris-HCl pH 7.5, 1 mM DTT
* Elution buffer II (to be made just before use): 2 M Urea, 50 mM Tris-HCl pH 7.5, 5 mM Iodoacetamide
* TMT Isobaric Label Reagent Set (Thermo ScientificTM)
* LC-MS grade H2O (Thermo Fisher Scientific; Cat# 10777404)
* TEABC (Sigma; Cat# 18597) – Make a 50 mM and 300 mM stock in LC-MS grade H2O, pH 8
* Mass Spec grade Trypsin (Promega, UK, Cat# V5111) – Make a stock by resuspending 20 µg trypsin in 0.05% (v/v) Acetic acid (just before use)
* LC-MS grade Methanol (MeOH) (Cat# 20847.307)
* LC-MS grade Acetonitrile (ACN) (Cat# 83640.320)
* Hydroxylamine (Sigma, Cat# 438227)
* Trifluoracetic acid (TFA) (Sigma; Cat# T6508)
* Formic acid (Sigma; Cat # 56302)
* Ammonium formate (Sigma, Cat #70221-25G-F)
* Ammonium hydroxide solution (Sigma, Cat# 338818-100 ML)
* 2-propanol
* Empore C18 disk (CDS analytical, Cat# 3M 2215)
* Empore Strong cation exchange (SCX) disks (CDS analyticals, Cat # 3M 2251)
* Millipore pH Strips (VWR # 1.09584.0001)
* LC vials
1. **Equipment**
* CO2 Incubator for cell culture maintained at 37 °C, 5% CO2 (v/v).
* Laminar flow hood for cell culture.
* Refrigerated bench-top centrifuge (Eppendorf microcentrifuge 5417R, or equivalent).
* Plate reader for Protein quantification (BioTek Epoch, or equivalent)
* Thermo mixer (Eppendorf ThermoMixer, or equivalent)
* SpeedVac Vacuum Concentrator (Thermo Scientific Cat #SPD140P1)
1. **Consumables**
* Tissue culture Petri dishes (100 mm) (BD Biosciences, Falcon®, catalog number: 351029)
* 1.5 ml low binding Eppendorf tubes (Sarstedt. REF# 72.706.600).
* Standard 1 ml and 200 µl Pipette tips (Greiner bio-one. Cat# 686271 and 685261 respectively).
* Stripetter/stripette gun and stripettes
* Set of Gilson pipettes P10, P200, P1000
* 15 ml and 50 ml Falcons
* 16-gauge needle (Sigma Aldrich Cat # Z261378)
* Glass pipettes (5 ml, 10 ml, 50 ml)
* C18 stage-tips (3 M Empore discs; C18 # 3M 2215 and SCX # 3M 2251)

**Steps:**

* 1. **Transient transfection of HEK293 cells**
		1. Plate cells in 10 cm dishes (one dish per experimental condition) to give a 60-70% confluency the following day (around 2.2 x 106 cells seeded per 10 cm dish).

Note: For cells stably expressing the GFP-tagged protein of interest, proceed to Step 1.2 (when cells are 90-100% confluent).

* + 1. Prepare a transfection mix in a sterile 1.5ml Eppendorf tube, containing (for each 10 cm dish):
* 3 µg FLAG-LRRK2 [Y1699C] plasmid
* 2 µg wild type RILPL1-GFP or 2 µg [R293A] RILPL1-GFP plasmid
* 1 µg HA-Rab8A [Q67L] plasmid
* 18 µl 1 mg/ml PEI Max 40K
* 500 µl OptiMem

1.1.3) Mix by vortexing and incubate at room temperature for 20 mins.

1.1.4) Add the mixture dropwise to the cells from step 1.1 using a P1000 sterile pipette.

1.1.5) Incubate cells at 37°C for 24 h.

* 1. **Preparation and quantification of cell lysates**

1.2.1 Remove culture medium completely from each dish using an aspirator.

1.2.2 Quickly rinse cells in the tissue culture dish by carefully pouring culture media without Foetal bovine serum (at room temperature) into the dish.

**Note:** As HEK293 cells are loosely attached to the dish surface, extra care should be taken during the washing step.

1.2.3 Pour off media from the culture dish and completely aspirate any residual media.

1.2.4 Immediately add 400 μl of ice-cold complete lysis buffer to each dish ensuring that the entire surface is covered by lysis buffer.

1.2.5 Transfer the plate on ice.

1.2.6 Scrape the cells on the dish using a cell lifter to ensure all cells are detached from the dish.

1.2.7 Using a pipette, transfer the lysate to a 1.5 mL Eppendorf tube.

1.2.8 Leave samples on ice for 20/30 minutes to allow for efficient lysis.

1.2.9 Spin down lysates at 17,000 g for 10 min at 4°C.

1.2.10 Transfer supernatant to a new Eppendorf tube and discard the pellet.

1.2.11 Proceed to estimating the protein concentration of cell lysates by Bradford assay according to the manufacturer’s instructions.

**Note: We recommend confirming the expression of the transiently expressed proteins by performing quantitative immunoblotting analysis as described in dx.doi.org/10.17504/protocols.io.bsgrnbv6.**

* 1. **Immunoprecipitation of GFP-tagged proteins from cell lysates**

1.3.1) Transfer n x 20 µl of ChromoTek GFP-Trap Agarose Beads (where n = number of samples) into a low binding Eppendorf tube.

1.3.2) Pellet the beads by centrifuging at 2500 g for 3 mins at 4°C.

1.3.3) Carefully aspirate the supernatant.

1.3.4) Resuspend the beads in 1 mL of IP wash buffer.

1.3.5) Repeat steps 1.3.2 to 1.3.4 twice.

1.3.6) Centrifuge at 2500 g for 3 mins at 4°C and aspirate the supernatant.

1.3.7) Resuspend beads from step 1.3.6 in n x 100 µl of IP wash buffer (where n = number of samples) to make a 1:1 slurry.

1.3.8) Aliquot the washed beads from step 1.3.7 into fresh low-binding Eppendorf tubes (40 μl of slurry for each sample, corresponding to 20 µl of ChromoTek GFP-Trap Agarose Beads). Leave the tubes on ice until use.

1.3.9) For each sample, transfer 500 µg lysate from step 1.2.10 to the washed beads.

1.3.10) Incubate for 2 hours at 4°C under mild agitation (on an orbital shaker).

1.3.11) Pellet the beads by centrifuging at 2500 g for 5 mins at 4°C.

1.3.12) Carefully aspirate the supernatant.

1.3.13) Resuspend the beads in 1 mL of IP wash buffer.

1.3.14) Repeat steps 1.3.11 to 1.3.13 twice.

1.3.15) Centrifuge at 2500 g for 5 mins at 4°C and aspirate the supernatant.

1.3.16) Immediately proceed to step 1.4.

* 1. **Elution and on-bead tryptic digestion of immunoprecipitated proteins**

1.4.1) Add 100 µl of **elution buffer** **I** to the beads from step 1.3.16.

1.4.2) Add 500 ng of sequencing grade trypsin to the mixture and incubate on a Thermomixer at 30°C 800 rpm for 30 minutes.

1.4.3) Centrifuge the mixture at 2500 g for 2 mins at room temperature. Collect the supernatant carefully into new Eppendorf tubes.

**Note:** Care must be taken for collecting the supernatant and not to collect the beads.

1.4.4) Add 100 µl of the **elution buffer II** is to the beads containing Eppendorf tubes and mix it gently by tapping. After that, collect the supernatant to the previous collection Eppendorf tube by centrifuging at 2,500 g for 2 minutes.

1.4.5) Incubate the Eppendorf tubes on the Thermomixer (30oC, 800 rpm) for overnight or minimum of 12 hr.

1.4.6) Add 1% (v/v) Trifluoroacetic Acid (TFA) to the digested peptides, incubate it for 5 mins at room temperature and centrifuge at 17000 g for 10 minutes.

* 1. **Peptide clean-up using C18 stage-tips**

1.5.1) Prepare a C18 stage-tip for each sample as described in dx.doi.org/10.17504/protocols.io.bs3tngnn.

A minimum of two discs are recommended for each 200 µl tip (assuming a peptide content of 5-10 µg).

1.5.2) C18 stage tips activation: add 80 µl of 100% ACN to each stage-tip and centrifuge at 2000 g for 1 min.

1.5.3) C18 stage tips equilibration: add 80 µl of 0.1% TFA to each stage-tip and centrifuge at 2000 g for 2 min.

1.5.4) Repeat step 1.5.3.

1.5.5) Transfer the C18 stage-tip to a new low-binding Eppendorf.

1.5.6) Load the peptides onto the C18 stage-tip from step 1.5.5 and centrifuge at 2000 g for 4 min.

1.5.7) Collect the flowthrough from step 1.5.6 and re-load onto the same C18 stage-tip. Centrifuge at 2000 g for 4 min.

1.5.8) Wash the C18 stage-tips by adding 80 µl of 0.1% TFA and centrifuging at 2000 g for 2 min.

1.5.9) Repeat step 1.5.8.

1.5.10) Transfer the C18 stage-tip to a new low-binding Eppendorf.

1.5.11) Add 30 µl of 30% (v/v) ACN in 0.1% (v/v) TFA to each stage-tip and centrifuge at 1000 g for 1 min.

1.5.12) Repeat step 1.5.11.

1.5.13) Take 1-2 µl of the digested peptides, vacuum dry and inject on MS to verify the digestion efficiency.

**Note:** Analyse data with a (1 h 10 mins) 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%.

1.5.13) Vacuum dry completely the remaining peptides and store at -80 oC until ready to undertake TMT labelling.

* 1. **Tandem Mass Tag Labelling**

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

1.6.2) Leave them at Room temperature for 10 mins. Following, vortex and briefly spin 2000 g for 2 mins.

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

1.6.3) 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.

1.6.4) Keep samples on a floater and place it on a water bath sonicator for 10 mins.

1.6.5) Centrifuge the samples at high speed (17000 g) for 10 mins at room temperature.

1.6.6) Transfer dissolved peptides into a 1.5 ml protein low binding Eppendorf tube.

1.6.7) Add 10 µL 20 µg/µl TMT reagent i.e. 200 µg aiming for a 1:1 mass ratio of peptide: TMT reagent.

1.6.8) Give a gentle vortex and brief spin at 2000 g for 1 min.

1.6.9) Place samples on a Thermomixer and incubate with a gentle agitation at 800 rpm, 2 hr room temperature.

1.6.10) Add another 50 µL 50 mM TEAB buffer to make a final 100 µL reaction. Vortex, brief spin at 2000 g for 10 mins and incubate on a Thermomixer for 10 mins.

**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.

1.6.11) 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 (2 h 25 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 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.

1.6.12) 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.

1.6.13) Thaw stored TMT labelled samples from step 1.6.12 to room temperature.

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

1.6.15) 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 20 mins.

1.6.16) Pool all samples into a single tube.

1.6.17) 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.

1.6.18) Snap freeze the remaining 880 µL reaction and vacuum dry using Speed vac, for the next step.

* 1. **Mini-basic RPLC fractionation**

To improve the proteomic coverage of TMT labelled interactome, we recommend performing a stage-tip based mini-bRP fractionation (as described in [1]) by performing the following steps.

1.7.1) Prepare four C18 stage-tips as described in dx.doi.org/10.17504/protocols.io.bs3tngnn.

1.7.2) Label eight 1.5 ml low-binding Eppendorf tubes as fraction 1 to fraction 8.

1.7.3) Prepare 50 mL of bRP stock solution (50 mM Ammonium formate in Milli-Q H2O).

1.7.4) Prepare Solvent A: Mix 20 ml of bRP stock solution with 20 ml of Milli-Q H2O (=25 mM Ammonium formate in Milli-Q H2O).

1.7.5) Prepare Solvent B: Mix 20 ml of bRP stock solution with 20 ml of 100% Acetonitrile (=25 mM Ammonium formate in 50% ACN).

1.7.6) Prepare elution solvents for fractionation (required in steps 1.7.22 and 1.7.23) as described in the table below.

Prepare each elution solvent in a 2 ml Eppendorf tube.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Elution solvent # (Fraction number) | Final ACN % in Elution solvent |  | Solvent A (ml) | Total volume (ml) |
| 8 | 100% | 100% ACN | 0 | N/A |
| 7 | 17.5% | 0.7 ml Solvent B (50% ACN) | 1.3 | 2.0 |
| 6 | 15.0% | 1.2 ml Elution solvent 7 (17.5% ACN) | 0.2 | 1.4 |
| 5 | 12.5% | 1.0 ml Elution solvent 6 (15.0% ACN) | 0.2 | 1.2 |
| 4 | 10.0% | 0.8 ml Elution solvent 5 (12.5% ACN) | 0.2 | 1.0 |
| 3 | 7.5% | 0.6 ml Elution solvent 4 (10.0% ACN) | 0.2 | 0.8 |
| 2 | 5.0% | 0.4 ml Elution solvent 3 (7.5% ACN) | 0.2 | 0.6 |
| 1 | 2.5% | 0.2 ml Elution solvent 2 (5.0% ACN) | 0.2 | 0.4 |

1.7.7) Dissolve peptides from step 1.6.6 in 200 µl of Solvent A.

**Note:** Check the pH of the samples using a pH strip. Adjust to pH 10 by adding 0.5 µl of 30% Ammonium hydroxide solution if necessary.

1.7.8) Place samples on a Thermomixer at 1800 rpm for 20 minutes at room temperature.

1.7.9) Centrifuge sample at 17,000g for 5 minutes at room temperature.

1.7.10) Transfer the supernatant into a new 1.5 ml protein low-binding Eppendorf tube.

1.7.11) Add 200 µl of 100% ACN to the C18 stage-tips (Step 1.7.1) and centrifuge at 2,500 g for 2 min at room temperature to activate the columns. Discard the flow through.

1.7.12) Add 200 µl of Solvent B to each column from step 1.7.11 and centrifuge at 2,500 g for 2 min at room temperature. Discard the flow through.

1.7.13) Add 200 µl of Solvent A to each column from step 1.7.12 and centrifuge at 2,500 g for 2 min at room temperature.

1.7.14) Transfer the column to a new low-binding Eppendorf tube.

1.7.15) Sample loading: Slowly load each sample (from step 1.7.10) onto a column (from step 1.7.14).

1.7.16) Centrifuge at 1500 g for 5 minutes at room temperature.

1.7.17) Collect the flowthrough from step 1.7.16 and slowly load onto the same column.

1.7.18) Centrifuge at 1500 g for 5 minutes at room temperature.

1.7.19) Transfer the column into a new 1.5 ml Eppendorf tube.

1.7.20) To wash the column, add 200 µl of Solvent A to the column and centrifuge at 2,500 g for 2 minutes at room temperature.

1.7.21) Transfer the column into the tube labelled as “Fraction 1” (from step 1.7.2)

1.7.22) Add 60 µl of Elution solvent 1 (from step 1.7.6) to the column and centrifuge at 1,500g for 2 min.

1.7.23) Repeat steps 1.7.21 and 1.7.22 to generate Fraction 2 to Fraction 8. For each fraction, add 60 µl of the corresponding Elution solvent (from step 1.7.6) to the column and centrifuge at 1,500g for 2 min.

1.7.24) Pool the 8 fractions from steps 1.7.22 and 1.7.23 as follows (to generate 4 final fractions): 1) Pool fraction 1 and 5; 2) Pool fraction 2 and 6; 3) Pool fraction 3 and 7; 4) Pool fraction 4 and 8.

1.7.25) Place fractions on dry ice and vacuum dry completely using a SpeedVac.

* 1. **LC-MS/MS analysis:**

1.8.1) Dissolve each fraction from step 1.7.25 in 20 µl of LC-buffer (3% (v/v) ACN, 0.1% (v/v) formic acid).

1.8.2) Place samples on a Thermomixer at 1800 rpm for 30 minutes at room temperature.

1.8.3) Transfer 10 µl of the sample from step 1.8.2 into a LC-vial for analysis (Step 1.8.4).

The remaining sample can be stored at -80°C as a back-up.

1.8.4) Perform LC-MS/MS analysis on an Orbitrap Lumos Tribrid mass spectrometer in MS3 mode. The mass spectrometer instrument settings in data acquisition are described in the table below.

|  |  |
| --- | --- |
| Application Mode |  Peptide |
| Method Duration (min) | 140 |
| Global Parameters |   |
| Infusion Mode |  Liquid Chromatography |
| Expected LC Peak Width (s) | 30 |
| Advanced Peak Determination |  False |
| Default Charge State | 2 |
| Internal Mass Calibration |  Off |
| Experiment#1 [MS] |   |
| Start Time (min) | 0 |
| End Time (min) | 140 |
| 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 |  Custom |
| Normalized AGC Target (%) | 50 |
| 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 |
| Data Dependent |   |
| Data Dependent Mode |  Number of Scans |
| Number of Dependent Scans | 10 |
| 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 (%) | 39 |
| Detector Type |  Orbitrap |
| Orbitrap Resolution | 30000 |
| Mass Range |  Normal |
| Scan Range Mode |  Auto |
| AGC Target |  Standard |
| Maximum Injection Time Mode |  Custom |
| Maximum Injection Time (ms) | 96 |
| Micro scans | 1 |
| Data Type |  Centroid |
| Use EASY-IC™ |  False |
| Scan Description |   |
| Filters |   |
| Precursor Selection Range |   |
| Selection Range Mode |  Mass Range |
| Mass Range (m/z) |  400-1200 |
| Precursor Ion Exclusion |   |
| Exclusion mass width |  ppm |
| Low | 25 |
| High | 25 |
| Isobaric Tag Loss Exclusion |   |
| Reagent |  TMT |
| 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 | 5 |
| MS Isolation Window (m/z) | 2 |
| MS2 Isolation Window (m/z) | 2 |
| Isolation Offset |  Off |
| Activation Type |  HCD |
| HCD Collision Energy (%) | 65 |
| Detector Type |  Orbitrap |
| Orbitrap Resolution | 50000 |
| Mass Range |  Normal |
| Scan Range Mode |  Define m/z range |
| Scan Range (m/z) |  100-500 |
| 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 |   |
| Number of Dependent Scans | 5 |

* + 1. The raw data was searched using MaxQuant version 1.6.6.0 using the parameters described below

|  |  |
| --- | --- |
| Parameter | Value |
| Version | 1.6.6.0 |
| User name | Rnirujogi |
| Machine name | SILAC-MRC0 |
| Date of writing | 10/23/2019 21:11:33 |
| Include contaminants | TRUE |
| PSM FDR | 0.01 |
| PSM FDR Crosslink | 0.01 |
| Protein FDR | 0.01 |
| Site FDR | 0.01 |
| Use Normalized Ratios For Occupancy | TRUE |
| Min. peptide Length | 7 |
| Min. score for unmodified peptides | 0 |
| Min. score for modified peptides | 40 |
| Min. delta score for unmodified peptides | 0 |
| Min. delta score for modified peptides | 6 |
| Min. unique peptides | 0 |
| Min. razor peptides | 1 |
| Min. peptides | 1 |
| Use only unmodified peptides and | TRUE |
| Modifications included in protein quantification | Oxidation (M);Acetyl (Protein N-term);Deamidation (NQ) |
| Peptides used for protein quantification | Razor |
| Discard unmodified counterpart peptides | TRUE |
| Label min. ratio count | 1 |
| Use delta score | FALSE |
| iBAQ | TRUE |
| iBAQ log fit | TRUE |
| Match between runs | TRUE |
| Matching time window [min] | 0.7 |
| Match ion mobility window [indices] | 0.05 |
| Alignment time window [min] | 20 |
| Alignment ion mobility window [indices] | 1 |
| Find dependent peptides | FALSE |
| Fasta file | D:\Database\HUMAN-Uniprot-150317\_Custom7.FASTA |
| Decoy mode | revert |
| Include contaminants | TRUE |
| Advanced ratios | TRUE |
| Fixed andromeda index folder |  |
| Temporary folder |  |
| Combined folder location |  |
| Second peptides | FALSE |
| Stabilize large LFQ ratios | FALSE |
| Separate LFQ in parameter groups | FALSE |
| Require MS/MS for LFQ comparisons | FALSE |
| Calculate peak properties | FALSE |
| Main search max. combinations | 200 |
| Advanced site intensities | FALSE |
| Write msScans table | TRUE |
| Write msmsScans table | TRUE |
| Write ms3Scans table | TRUE |
| Write allPeptides table | TRUE |
| Write mzRange table | TRUE |
| Write pasefMsmsScans table | TRUE |
| Write accumulatedPasefMsmsScans table | TRUE |
| Max. peptide mass [Da] | 4600 |
| Min. peptide length for unspecific search | 8 |
| Max. peptide length for unspecific search | 25 |
| Razor protein FDR | TRUE |
| Disable MD5 | FALSE |
| Max mods in site table | 3 |
| Match unidentified features | FALSE |
| Epsilon score for mutations |  |
| Evaluate variant peptides separately | TRUE |
| Variation mode | None |
| MS/MS tol. (FTMS) | 20 ppm |
| Top MS/MS peaks per Da interval. (FTMS) | 12 |
| Da interval. (FTMS) | 100 |
| MS/MS deisotoping (FTMS) | TRUE |
| MS/MS deisotoping tolerance (FTMS) | 7 |
| MS/MS deisotoping tolerance unit (FTMS) | ppm |
| MS/MS higher charges (FTMS) | TRUE |
| MS/MS water loss (FTMS) | TRUE |
| MS/MS ammonia loss (FTMS) | TRUE |
| MS/MS dependent losses (FTMS) | TRUE |
| MS/MS recalibration (FTMS) | FALSE |
| MS/MS tol. (ITMS) | 0.5 Da |
| Top MS/MS peaks per Da interval. (ITMS) | 8 |
| Da interval. (ITMS) | 100 |
| MS/MS deisotoping (ITMS) | FALSE |
| MS/MS deisotoping tolerance (ITMS) | 0.15 |
| MS/MS deisotoping tolerance unit (ITMS) | Da |
| MS/MS higher charges (ITMS) | TRUE |
| MS/MS water loss (ITMS) | TRUE |
| MS/MS ammonia loss (ITMS) | TRUE |
| MS/MS dependent losses (ITMS) | TRUE |
| MS/MS recalibration (ITMS) | FALSE |
| MS/MS tol. (TOF) | 40 ppm |
| Top MS/MS peaks per Da interval. (TOF) | 10 |
| Da interval. (TOF) | 100 |
| MS/MS deisotoping (TOF) | TRUE |
| MS/MS deisotoping tolerance (TOF) | 0.01 |
| MS/MS deisotoping tolerance unit (TOF) | Da |
| MS/MS higher charges (TOF) | TRUE |
| MS/MS water loss (TOF) | TRUE |
| MS/MS ammonia loss (TOF) | TRUE |
| MS/MS dependent losses (TOF) | TRUE |
| MS/MS recalibration (TOF) | FALSE |
| MS/MS tol. (Unknown) | 0.5 Da |
| Top MS/MS peaks per Da interval. (Unknown) | 8 |
| Da interval. (Unknown) | 100 |
| MS/MS deisotoping (Unknown) | FALSE |
| MS/MS deisotoping tolerance (Unknown) | 0.15 |
| MS/MS deisotoping tolerance unit (Unknown) | Da |
| MS/MS higher charges (Unknown) | TRUE |
| MS/MS water loss (Unknown) | TRUE |
| MS/MS ammonia loss (Unknown) | TRUE |
| MS/MS dependent losses (Unknown) | TRUE |
| MS/MS recalibration (Unknown) | FALSE |
| Site tables | Deamidation (NQ)Sites.txt;Oxidation (M)Sites.txt;Phospho (STY)Sites.txt |

1. Ruprecht, B., et al., *High pH Reversed-Phase Micro-Columns for Simple, Sensitive, and Efficient Fractionation of Proteome and (TMT labeled) Phosphoproteome Digests.* Methods Mol Biol, 2017. **1550**: p. 83-98.
2. dx.doi.org/10.17504/protocols.io.bs3tngnn
3. **dx.doi.org/10.17504/protocols.io.bsgrnbv6**