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

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We use this protocol and it's working

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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.

Attachments



[710-1531.docx](#)

50KB

Guidelines

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

Reagents

A. For cell culture, transient transfection and GFP immunoprecipitation:

-  HEK293 **ATCC Catalog #CRL-1573** cultured in complete growth medium.
- **Growth medium:** Dulbecco's Modified Eagle's Medium (DMEM), High Glucose, no glutamine (Gibco™, catalog number: 11960044, or equivalent) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Sigma #F7524, or equivalent), 2 mM L-glutamine (Gibco™, catalog number: 25030024, or equivalent), Penicillin-Streptomycin 100U/mL (Gibco™, catalog number: 15140122, or equivalent).
-  Trypsin-EDTA 0.05% phenol red **Gibco - Thermo Fisher Scientific Catalog #25300054** (or equivalent)
-  DPBS no calcium no magnesium **Gibco - Thermo Fisher Scientific Catalog #14190169**
- Linear polyethylenimine (

 PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40000) **Polysciences, Inc. Catalog #24765-1**

); 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) **Thermo Fisher Scientific Catalog #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 **Proteintech Catalog # gta-20**
- **Lysis Buffer:**

A
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)



A
1X phosSTOP phosphatase inhibitor cocktail (PhosSTOP tablet: Roche, REF# 04906837001; to be added just before use)
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

B. For TMT mass spectrometry analysis:

-  DL-Dithiothreitol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0632-10G** – Prepare fresh as a 100 mM Stock in Milli-Q H₂O
-  Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I1149** – Prepare fresh as a 200 mM stock in Milli-Q H₂O
-  Urea **Thermo Fisher Catalog #29700**

- **Elution buffer I** (to be made just before use):

A	B
Urea	2 M
Tris-HCl pH 7.5	50 mM
DTT	1 mM

- **Elution buffer II** (to be made just before use):

A	B
Urea	2 M
Tris-HCl pH 7.5	50 mM
Iodoacetamide	5 mM

- TMT Isobaric Label Reagent Set (Thermo Scientific™)
-  LC-grade Water **Fisher Scientific Catalog #10777404**
-  Triethylammonium bicarbonate buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #18597** – Make a 50 mM and 300 mM stock in LC-MS grade H₂O, pH 8



-  Seq Grade Modified Trypsin, 100ug (5 × 20ug) **Promega Catalog #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 solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #438227**
-  Trifluoroacetic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T6508**
- Formic acid (Sigma; Cat # 56302)
-  Ammonium formate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #70221-25G-F**
-  Ammonium hydroxide solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #338818**
- 2-propanol
-  C18-HD bonded silica 12um particle size 47mm. **CDS Analytical LLC Catalog #2215**
-  Empore Disk Strong Cation Exchange 12um particle size 47mm **CDS Analytical LLC Catalog #Empore 2251**
-  pH indicator strips mid range **VWR International (Avantor) Catalog #1.09584.0001**
- LC vials

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)

Equipment

Savant™ SpeedVac™ Medium Capacity Vacuum Concentrators for Combinatorial Chemistry^{NAM} Applications^E

SpeedVac Vacuum Concentrator

TYPE

Thermo Scientific™

BRAND

SPD140P1

SKU

<https://www.thermofisher.com/order/catalog/product/SPD140P1-115>

LINK



Consumables

-  Falcon® 100 mm x 15 mm Not TC-treated Bacteriological Petri Dish 20/Pack 500/Case Sterile **Corning Catalog #351029**
-  SafeSeal reaction tube 1.5 ml PP PCR Performance Tested Low protein-binding **Sarstedt Catalog #72.706.600**
-  Greiner Bio-One™ Polypropylene Pipette Tip **Fisher Scientific Catalog #686271** and  PIPETTE TIP 10 - 100 µL SUITABLE FOR EPPENDORF 96 PIECES / ST RACK **greiner bio-one Catalog #685261**
- .
- Stripetter/stripette gun and stripettes
- Set of Gilson pipettes P10, P200, P1000
- 15 ml and 50 ml Falcons
-  Stainless steel 316 syringe needle pipetting blunt 90° tip **Merck MilliporeSigma (Sigma-Aldrich) Catalog #Z261378**
- Glass pipettes (5 ml, 10 ml, 50 ml)
- C18 stage-tips (3 M Empore discs; C18 # 3M 2215 and SCX # 3M 2251)

 DMEM high glucose no glutamine **Thermo Fisher Scientific Catalog #11960044**

 Fetal Bovine Serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #F7524**

 L-Glutamine (200mM) **Thermo Fisher Scientific Catalog #25030024**

 Penicillin-Streptomycin (10,000 U/mL) **Gibco - Thermo Fisher Scientific Catalog #15140122**

 NP-40 Alternative **Merck Millipore (EMD Millipore) Catalog #492016**

 Roche PhosSTOP™ **Merck MilliporeSigma (Sigma-Aldrich) Catalog #4906837001**

 cComplete™ EDTA-free Protease Inhibitor Cocktail **Roche Catalog #11873580001**



Transient transfection of HEK293 cells

- 1 Plate cells in 10 cm dishes (one dish for each experimental condition) to give a 60-70% confluency the following day (around 2.2×10^6 cells seeded per 10 cm dish).

Note

Note: For cells stably expressing the GFP-tagged protein of interest, proceed to **Preparation and quantification of cell lysates** (when cells are 90-100% confluent).

- 2 Prepare a transfection mix in a sterile 1.5ml Eppendorf tube, containing (for each 10 cm dish):

A	B
FLAG-LRRK2 [Y1699C] plasmid	3 µg
wild type RILPL1-GFP or 2 µg [R293A] RILPL1-GFP plasmid	2 µg
HA-Rab8A [Q67L] plasmid	1 µg
1 mg/ml PEI Max 40K	18 µl
OptiMem	500 µl

- 3 Mix by vortexing and incubate at Room temperature for 00:20:00 .

20m



- 4 Add the mixture dropwise to the cells from step 1 using a P1000 sterile pipette.



- 5 Incubate cells at 37 °C for 24:00:00 .

1d



Preparation and quantification of cell lysates

- 6 Remove culture medium completely from each dish using an aspirator.



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



Note

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

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

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



- 10 Transfer the plate  On ice .

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

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



- 13 Leave samples  On ice for 20-30 minutes to allow for efficient lysis.

- 14 Spin down lysates at  17000 x g, 4°C, 00:10:00 .

10m



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

- 16 Proceed to estimating the protein concentration of cell lysates by Bradford assay according to the manufacturer's instructions.



Note

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](https://doi.org/10.17504/protocols.io.bsgrnbv6).

Immunoprecipitation of GFP-tagged proteins from cell lysates

- 17 Transfer $n \times 20 \mu\text{L}$ of packed ChromoTek GFP-Trap Agarose Beads (where n = number of samples) into a low binding Eppendorf tube. 
- 18 Pellet the beads by centrifuging at $2500 \times g$, 4°C , 00:03:00.  3m
- 19 Carefully aspirate the supernatant.
- 20 Resuspend the beads in 1 mL of IP wash buffer.
- 21 Repeat steps 18 to 20 twice.
- 22 Centrifuge at $2500 \times g$, 4°C , 00:03:00 and aspirate the supernatant.  3m
- 23 Resuspend beads from step 22 in $n \times 20 \mu\text{L}$ of IP wash buffer (where n = number of samples) to make a 1:1 slurry.
- 24 Aliquot the washed beads from step 23 into fresh low-binding Eppendorf tubes ($40 \mu\text{L}$ of slurry for each sample, corresponding to $20 \mu\text{L}$ of packed ChromoTek GFP-Trap Agarose Beads). Leave the tubes  On ice until use.
- 25 For each sample, transfer $500 \mu\text{g}$ lysate from step 15 to the washed beads. 



- 26 Incubate for 02:00:00 at 4 °C under mild agitation (on an orbital shaker). 2h
- 27 Pellet the beads by centrifuging at 2500 x g, 4°C, 00:05:00 . 5m
- 28 Carefully aspirate the supernatant.
- 29 Resuspend the beads in 1 mL of IP wash buffer.
- 30 Repeat steps 27 to 29 twice.
- 31 Centrifuge at 2500 x g, 4°C, 00:05:00 and aspirate the supernatant. 5m
- 32 Immediately proceed to **Elution and on-bead tryptic digestion of immunoprecipitated proteins** .

Elution and on-bead tryptic digestion of immunoprecipitated proteins

- 33 Add 100 µL of **elution buffer I** to the beads from step 32.
- 34 Add 500 ng of sequencing grade trypsin to the mixture and incubate on a Thermomixer at 800 rpm, 30°C, 00:30:00 .
- 35 Centrifuge the mixture at 2500 x g, Room temperature, 00:02:00 . Carefully transfer the supernatant to new Eppendorf tubes, being careful not to disturb the beads. 2m
- 36 Add 100 µL of **elution buffer II** to the beads from step 35 and mix gently by tapping. Centrifuge the mixture at 2500 x g, Room temperature, 00:02:00 . Carefully transfer the supernatant to the collection Eppendorf tubes from step 35, being careful not to disturb the beads. 2m



- 37 Incubate the Eppendorf tubes on the Thermomixer  800 rpm, 30°C  Overnight   2h
- 38 Add 1% (v/v) Trifluoroacetic Acid (TFA) to the digested peptides from step 37. Incubate for  00:05:00 at  Room temperature and centrifuge at  17000 x g, 00:10:00   15m

Peptide clean-up using C18 stage-tips

- 39 Prepare a C18 stage-tip for each sample as described in dx.doi.org/10.17504/protocols.io.bs3tnngn.

Note

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

- 40 **C18 stage tips activation:** add  80 µL of 100% ACN to each stage-tip and centrifuge at  2000 x g, 00:01:00 .   1m
- 41 **C18 stage tips equilibration:** add  80 µL of 0.1% TFA to each stage-tip and centrifuge at  2000 x g, 00:02:00 .   2m
- 42 Repeat step 41.
- 43 Transfer the C18 stage-tip to a new low-binding Eppendorf.
- 44 Load the digested peptides from step 38 onto the C18 stage-tip from step 43 and centrifuge at  2000 x g, 00:04:00 .  4m



45 Collect the flowthrough from step 44 and re-load onto the same C18 stage-tip.
Centrifuge at  2000 x g, 00:04:00 .

4m



46 Wash the C18 stage-tips by adding  80 μL of 0.1% TFA and centrifuging at
 2000 x g, 00:02:00 .

2m



47 Repeat step 46.

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



49 Add  30 μL of 30% (v/v) ACN in 0.1% (v/v) TFA to each stage-tip and centrifuge at
 1000 x g, 00:01:00 .

1m



50 Repeat step 49.

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

Note

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%.

52 Vacuum dry completely the remaining peptides and store at  -80 °C until ready to undertake TMT labelling.

Tandem Mass Tag Labelling



53 Dissolve  800 μg of each of the TMT mass tag reagents within the 11-plex TMT reagent kit in  40 μL of 100% anhydrous acetonitrile to obtain a  20 $\mu\text{g}/\mu\text{L}$ concentration for each TMT reporter tag.

54 Leave at  Room temperature for  00:10:00 , then vortex and spin  2000 x g, 00:02:00 .

12m



Note

Note: Dissolved TMT reagents are prone to hydrolysis. Once reconstituted, aliquot and immediately transfer to  -80 $^{\circ}\text{C}$ for storage (up to six months). Avoid multiple freeze thaw cycles.

55 Dissolve lyophilized peptides from step 52 in  50 μL of a mixture containing  38 μL  50 millimolar (mM) TEAB buffer +  8 μL 100% (by vol) anhydrous acetonitrile.

Note

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

56 Place the samples in a water bath sonicator for  00:10:00 .

10m

57 Centrifuge the samples  17000 x g, 00:10:00 .

10m



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

59 Add  10 μL  20 $\mu\text{g}/\mu\text{L}$ TMT reagent i.e.  200 μg aiming for a 1:1 mass ratio of peptide: TMT reagent.





60 Give a gentle vortex and spin at  2000 x g, 00:01:00 .

1m



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



62 Add another  50 μL  50 millimolar (mM) TEAB buffer to make a final  100 μL reaction. Vortex, brief spin at  2000 x g, 00:10:00 and incubate on a Thermomixer for  00:10:00 .

20m



Note

Note: It is good practice to maintain the total volume to  100 μL final reaction as this helps reducing pipetting error when aliquoting  5 μL of sample for label check efficiency.

63 In order to verify the TMT labelling efficiency of each TMT mass tag, take a  5 μL aliquot from each of the TMT-labelled samples and pool the aliquots in a single tube. Vacuum dry immediately using a SpeedVac. Store the remaining  95 μL at  -80 °C until the labelling efficiency has been verified.

Note

Note: It is important to verify the labelling efficiency of each TMT mass tag and it should label > 98%, by analysing on Mass spec. We recommend doing this employing a (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.

64 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.



- 65 Thaw stored TMT labelled samples from step 63 to Room temperature .
- 66 Prepare 5% (by vol) final Hydroxyl amine solution by dissolving in water from a 50% (by vol) stock solution.
- 67 Add 5 μ L 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 .
- 68 Pool all samples into a single tube.
- 69 Transfer 20% of the reaction to a new low-binding Eppendorf tube as a backup: snap freeze on dry ice and vacuum dry.

20m

**Note**

Note: This can be used in case of sample loss during the downstream analysis or for further validation.

- 70 Snap freeze the remaining 80% of the reaction and vacuum dry using Speed vac.

Mini-basic RPLC fractionation

71

Note

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.

Prepare four C18 stage-tips as described in dx.doi.org/10.17504/protocols.io.bs3tnngnn.

- 72 Label eight 1.5ml low-binding Eppendorf tubes as "Fraction 1" to "Fraction 8".

- 73 Prepare  50 mL of bRP stock solution ( 50 millimolar (mM) Ammonium formate in Milli-Q H₂O).
- 74 **Prepare Solvent A:** Mix  20 mL of bRP stock solution with  20 mL of Milli-Q H₂O (=  25 millimolar (mM) Ammonium formate in Milli-Q H₂O).
- 75 **Prepare Solvent B:** Mix  20 mL of bRP stock solution with  20 mL of 100% Acetonitrile (=  25 millimolar (mM) Ammonium formate in 50% ACN).
- 76 Prepare elution solvents for fractionation (required in steps 92 and 93) as described in the table below.
Prepare each elution solvent in a 2 ml Eppendorf tube.

A	B	C	D	E
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



	A	B	C	D	E
	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

77 Dissolve peptides from step 58 in  200 μL of Solvent A.

Note

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

78 Place samples on a Thermomixer at  1800 rpm, Room temperature , 00:20:00 .

79 Centrifuge sample at  17000 x g, Room temperature, 00:05:00 .

5m



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

81 Add  200 μL of 100% ACN to the C18 stage-tips (Step 71) and centrifuge at  2500 x g, Room temperature, 00:02:00 to activate the columns. Discard the flow through.

2m



82 Add  200 μL of Solvent B to each column from step 81 and centrifuge at  2500 x g, Room temperature, 00:02:00 . Discard the flow through.

2m



83 Add  200 μL of Solvent A to each column from step 82 and centrifuge at  2500 x g, Room temperature, 00:02:00 .

2m





- 84 Transfer the column to a new low-binding Eppendorf tube.
- 85 **Sample loading:** Slowly load each sample (from step 80) onto a column (from step 84).
- 86 Centrifuge at  1500 x g, Room temperature, 00:05:00 . 
- 87 Collect the flowthrough from step 86 and slowly load onto the same column.
- 88 Centrifuge at  1500 x g, Room temperature, 00:05:00 . 
- 89 Transfer the column into a new 1.5ml Eppendorf tube.
- 90 To wash the column, add  200 μL of Solvent A to the column and centrifuge at  2500 x g, Room temperature, 00:02:00 . 

- 91 Transfer the column into the tube labelled as "Fraction 1" (from step 72).
- 92 Add  60 μL of Elution solvent 1 (from step 76) to the column and centrifuge at  1500 x g, 00:02:00 . 

- 93 Repeat steps 91 and 92 to generate Fraction 2 to Fraction 8. For each fraction, add 60 μL of the corresponding Elution solvent (from step 76) to the column and centrifuge at  1500 x g, 00:02:00 . 


- 94 Pool the 8 fractions from steps 92 and 93 as follows (to generate 4 final fractions):
 - Pool fraction 1 and 5;
 - Pool fraction 2 and 6;
 - Pool fraction 3 and 7;
 - Pool fraction 4 and 8.
- 95 Place fractions on dry ice and vacuum dry completely using a SpeedVac.

LC-MS/MS analysis

- 96 Dissolve each fraction from step 95 in  20 µL of LC-buffer (3% (v/v) ACN, 0.1% (v/v) formic acid).
- 97 Place samples on a Thermomixer at  1800 rpm, Room temperature , 00:30:00 .
- 98 Transfer  10 µL of the sample from step 97 into a LC-vial for analysis (Step 99). The remaining sample can be stored at  -80 °C as a back-up.
- 99 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.

	A	B
	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



A	B
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



A	B
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	
ddMS ² 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



A	B
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	
MS ⁿ Level	3
Synchronous Precursor Selection	True
Number of SPS Precursors	5
MS Isolation Window (m/z)	2
MS2 Isolation Window (m/z)	2



A	B
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

100 The raw data was searched using MaxQuant version 1.6.6.0 using the parameters described below.

A	B
Parameter	Value
Version	1.6.6.0



A	B
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



A	B
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



A	B
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



A	B
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



A	B
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

Protocol references

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.bs3tnn
3. dx.doi.org/10.17504/protocols.io.bsgrnbv6