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

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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

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:

Keywords:
Immunoprecipitation of GFP-tagged proteins from cell lysates, Tandem Mass Tag (TMT) Mass Spectrometry Analysis

- 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 Fischer Catalog #25300054** (or equivalent)
- DPBS no calcium no magnesium **Gibco - Thermo Fischer 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 H_2O, 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)
1X phosSTOP phosphatase inhibitor cocktail (PhosSTOP tablet: Roche, REF# 04906837001; to be added just before use)

	A
	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 H_2O
- 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^{TM})
- 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 H_2O, pH 8
- Seq Grade Modified Trypsin, 100ug (5 x 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) Catalo #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 Analytic LLC Catalog #Empore 2251**
- pH indicator strips mid range **VWR International Catalog #1.09584.0001**
- LC vials

Equipment

- CO_2 Incubator for cell culture maintained at 37 °C, 5% CO_2 (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 Applications	NAME
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 MilliporeSigm (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 Catalog #15140122**

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

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

cOmplete™ 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 x 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 µL 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.

Immunoprecipitation of GFP-tagged proteins from cell lysat..

17 Transfer n x 20 µL 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 x 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 x g, 4°C, 00:03:00 and aspirate the supernatant. 3m

23 Resuspend beads from step 22 in n x 20 µL 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 µL of slurry for each sample, corresponding to 20 µL of packed ChromoTek GFP-Trap Agarose Beads). Leave the tubes On ice until use.

25 For each sample, transfer 500 µg 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 immunoprecipitate...

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 (or minimum of 12 hr). 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.bs3tngnn**.

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 1m

2000 x g, 00:01:00 .

41 **C18 stage tips equilibration**: add 80 µL of 0.1% TFA to each stage-tip and centrifuge at 2m

2000 x g, 00:02:00 .

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 4m

2000 x g, 00:04:00 .

45 Collect the flowthrough from step 44 and re-load onto the same C18 stage-tip. Centrifuge at 4m

2000 x g, 00:04:00 .

46 Wash the C18 stage-tips by adding 80 µL of 0.1% TFA and centrifuging at 2m

2000 x g, 00:02:00 .

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 µg/µL 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 °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 [M] 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 µg/µL 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 [M] 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 µL 5% (by vol) Hydroxylamine to each sample to quench TMT reaction by incubating the reaction at Room temperature on a Thermomixer for 00:20:00. 20m

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.

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.bs3tngnn**.

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

73 Prepare 50 mL of bRP stock solution ([M] 50 millimolar (mM) Ammonium formate in Milli-Q H_2O).

74 **Prepare Solvent A**: Mix 20 mL of bRP stock solution with 20 mL of Milli-Q H_2O (= [M] 25 millimolar (mM) Ammonium formate in Milli-Q H_2O).

75 **Prepare Solvent B**: Mix 20 mL of bRP stock solution with 20 mL of 100% Acetonitrile (= [M] 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
	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 pH 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 2m

2500 x g, Room temperature, 00:02:00 to activate the columns. Discard the flow through.

82 Add 200 µL of Solvent B to each column from step 81 and centrifuge at 2m

2500 x g, Room temperature, 00:02:00 . Discard the flow through.

83 Add 200 µL of Solvent A to each column from step 82 and centrifuge at 2m

2500 x g, Room temperature, 00:02:00 .

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. 5m

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. 5m

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. 2m

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. 2m

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. 2m

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

	A	B
	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

	A	B
	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

	A	B
	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	
	MS^n Level	3
	Synchronous Precursor Selection	True

A	B
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

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
User name	Rnirujogi

	A	B
	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

	A	B
	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

	A	B
	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

	A	B
	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