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Identification of PKC-regulated phosphosites on LRRK1 by mass spectrometry analysis

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asap

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ABSTRACT

We describe a non-radioactive, mass spectrometry-based assay that we deploy for identifying novel PKC-regulated sites on LRRK1 that are responsible for activation of its kinase activity.

ATTACHMENTS

457-966.docx

DOI

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KEYWORDS

PKC-regulated phosphosites , LRRK1 , Mass spectrometry analysis

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MATERIALS TEXT

MATERIALS

Reagents:

- Recombinant PKC protein (available from MRC Reagents and Services: https://mrcppureagents.dundee.ac.uk/)
- Recombinant LRRK1 wild type [and kinase inactive? D1409A, 27-2015] protein

Recombinant LRRK1 protein is expressed and purified by following the protocol described in: **XXXXX**

Kinase assay buffer:

A	B
HEPES pH 7.5	25 mM
2-mercaptoethanol	0.1% (v/v)
KCl	50 mM
CaCl2	1 mM
MgCl2	10 mM
ATP	1 mM

- L-α-Phosphatidylserine (Avanti Polar Lipids, resuspended in methanol and chloroform at a 1:1 ratio for long-term storage)
- L-α-Diacylglyerol (Avanti Polar Lipids, resuspended in methanol and chloroform at a 1:1 ratio for long-term storage)

4X Loading buffer:

NUPAGE LDS sample buffer (4x) **Thermo Fisher Scientific Catalog #NP0007** or 4X SDS loading buffer:

A	B
Tris-HCl pH6.8	250mM
SDS	8% (w/v)
Glycerol	40% (v/v)
Bromophenol blue	0.02% (w/v)

SDS-PAGE buffer:

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- For NuPAGE™ Bis-Tris gels:

  ☒ NuPAGE™ MOPS SDS Running Buffer (20X) **Thermo Fisher Catalog #NP000102**)
- For self-cast Bis-Tris gels:

A	B
MOPS	50 mM
Tris	50 mM
SDS	0.1% (w/v)
EDTA	1 mM

☒ InstantBlue® Coomassie Protein Stain (ISB1L)

- (ab119211) **Abcam Catalog #119211** , or equivalent

  ☒ DL-Dithiothreitol (DTT) **Sigma**
- **Aldrich Catalog #43815**

  ☒ Ammonium bicarbonate **Sigma**
- **Aldrich Catalog #A6141**

  ☒ Acetonitrile ≥99.9% **VWR**
- **Avantor Catalog #1.00030.2500**

  ☒ Iodoacetamide **Millipore**
- **Sigma Catalog #I1149**

  ☒ Trifluoroacetic acid for HPLC > 99.0% **Sigma-**
- **aldrich Catalog #302031-100ML**

Prepare a 20% (by vol) aqueous trifluroacetic acid (TFA) stock and store at **4 °C** .

☒ Seq Grade Modified Trypsin, 100ug (5 x

- 20ug) **Promega Catalog #V5111**).

  ☒ Chymotrypsin, Sequencing Grade,
- 25ug **Promega Catalog #V1061**)

  ☒ Asp-N, Sequencing Grade,
- 2ug **Promega Catalog #V1621**

Store protease stocks at **-20 °C** and thaw **On ice** ? just before the digestion step.

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NuPAGE 4–12% Bis–Tris Midi Gel **Thermo Fisher Scientific Catalog #WG1403BOX**

NuPAGE™ 4 to 12% Bis-Tris 1.0 mm Midi Protein Gels **Thermo Fisher Scientific Catalog #WG1402BOX**

ReproSil-Pur C18 1.9 µm beads **Contributed by users Catalog #EV1113** .

Equipment:

Eppendorf® microcentrifuge
Centrifuge
Eppendorf® **5417**

, or equivalent

Savant SpeedVac system
Vacuum concentrator
Thermo Fisher Scientific **SPD140DDA**

, or equivalent

- Thermo mixer (Eppendorf ThermoMixer, or equivalent)
- Disposable Glass Culture Tubes (Fisherbrand Round Bottom Disposable Borosilicate Glass Tubes, or equivalent)
- XCell4 SureLock Midi-Cell Electrophoresis System (if using Invitrogen NuPAGE precast midi gels), or equivalent gel electrophoresis apparatus.

See-saw rocker
Rocker
VWR **SSL4**

, or equivalent

Eppendorf® LoBind microcentrifuge tubes
Microcentrifuge tubes
Eppendorf **022431081**

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16-gauge needle
Needle

Sigma Aldrich **Z261378**

Spray duster
Pressurised, HFC free air duster perfect for keyboards, cameras and difficult to reach areas. Ozone Friendly

Qconnect **KFO4499**

- PTFE-O rings

Place the PTFE-O-ring on top of the Eppendorf tube to serve as an adaptor such a way that 3/4th of the Stage-tip could be placed into the tube during the centrifugation step. PTFE-O-rings can be purchased from NEST group desalting columns and re use them https://www.nestgrp.com/.

- X72 40 mL Amber class EPA vial W Cap and seal (Cole Parmer # 10572553)

CDS Analytical 2215 Empore™ C-18 Disk, 47mm; 60/PK
Empore organic SPE disks are ideal for solid phase extraction of large water samples.

Cole Parmer **2215**

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** , punch 2 or 3 layers with 16-guage needle.

- Exploris 240 Mass spectrometer.
- EvoSep Liquid chromatrography system.

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Any nano-LC such as Easy nLC or Ultimate 3000 Dionex can be used instead.

- Proteome Discoverer 2.4 software suite with SEQUEST or Mascot search algorithm.

Preparation of lipid vesicles for PKC activation

1 

Clean a disposable glass culture tube by washing three times with 100% methanol. Allow to air-dry.

2 

Pipette **0.5 µL** of Diacylglycerol (stock concentration is **10 mg/mL**) and **5 µL** of Phosphatidylserine (stock concentration is **10 mg/mL**) into the cleaned and dried glass tube.

These quantities will provide sufficient lipid vesicles for 25 reactions at a volume of **20 µL** per reaction.

3 Vacuum dry lipids using a SpeedVac system for **00:10:00** . This should leave a visible, translucent lipid pellet. 10m

Ensure that lipids are completely dried as any residual chloroform or methanol will inhibit the kinase reaction.

4 

Resuspend lipids from step 3 in **50 µL** of **25 millimolar (mM)** HEPES **7.4** , **50 millimolar (mM)** KCl. Vortex gently until pellet is no longer visible.

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Kinase Reaction: Phosphorylation of LRRK1 by PKC

5 Prepare a primary “2X master mix” containing **50 millimolar (mM)** HEPES **pH 7.5**, **100 millimolar (mM)** KCl, 0.2% (v/v) 2-Mercaptoethanol, **20 millimolar (mM)** $MgCl_2$, **2 millimolar (mM)** ATP, **2 millimolar (mM)** $CaCl_2$, **200 µg/ml** Phosphatidylserine and **20 µg/ml** Diacylglycerol.

6

For each reaction, add **15 µL** of the primary “2X master mix” to a clean Eppendorf tube.

7 5m

Add **7.5 µL** of **200 nanomolar (nM)** LRRK1 wild type protein (final concentration is **50 nanomolar (nM)**) to each reaction and allow equilibration **On ice** for **00:05:00** .

8

Start the kinase reaction by adding **7.5 µL** of **400 nanomolar (nM)** PKC Alpha protein (final concentration is **100 nanomolar (nM)**).

The final reaction volume should be **30 µL** .

Reactions not including PKC Alpha are also included as a negative control to identify phosphorylation sites that are only present when recombinant LRRK1 protein is incubated with PKC Alpha. In these reactions, add **7.5 µL** of **25 millimolar (mM)** HEPES **pH 7.4**, **50 millimolar (mM)** KCl instead of PKC Alpha protein.

9 45m

Transfer the Eppendorf tubes to the thermo mixer set at **30 °C** , **1000 rpm** . Incubate for **00:45:00** .

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10

Stop the kinase reaction by adding **10 µL** of 4X LDS loading buffer to the reaction mix to a final concentration of 1X.

11 5m

Incubate the samples for **00:05:00** at **70 °C** on a heat block before proceeding to **SDS-polyacrylamide gel electrophoresis (SDS-PAGE) section.**

SDS-polyacrylamide gel electrophoresis (SDS-PAGE):

12

Load samples onto a NuPAGE 4–12% Bis–Tris Midi Gel (ThermoFisherScientific, Cat#WG1402BOX or Cat#WG1403BOX), alongside pre-stained molecular weight markers (ranging from 10 kDa to 250 kDa). Rinse wells carefully with running buffer before loading samples.

Load the complete reaction onto gels to ensure detection of proteins by Instant Blue stain.

13 2h

Electrophorese samples at 130V with MOPS SDS running buffer for **02:00:00** or until the blue dye runs off the gel.

14 1h

Place gel in a clean glass 15 cm dish and cover with **15 mL** - **20 mL** of InstantBlue® Coomassie Protein stain. Incubate on see-saw rocker for **01:00:00** at **Room temperature** .

15 2m

Replace the InstantBlue® Protein stain with double distilled water and allow to de-stain at **Room temperature** **Overnight** before proceeding with peptide digestion as described in **Total Protein Digestion section.**

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Total Protein Digestion

16 Using a clean scalpel, excise stained-bands corresponding to LRRK1 from gel and cut into approximately 1mm^2 gel pieces.

17 Transfer the gel pieces into a low-bind tube.

18  10m

De-stain gel pieces by repeated **00:10:00** washes in 40% (v/v) ACN in **40 millimolar (mM)** NH_4HCO_3.

Wash by incubation on thermomixer set to **1200 rpm** at **Room temperature** . Repeat step 18 until gel pieces are completely colorless.

19  30m

Reduce peptides by addition of **100 µL** of **5 millimolar (mM)** DTT in **40 millimolar (mM)** NH_4HCO_3. Incubate on thermomixer at **56 °C** for **00:30:00** , **1200 rpm** .

20  10m

Remove the DTT solution and incubate gel pieces in 40% (v/v) ACN in **40 millimolar (mM)** NH_4HCO_3 for **00:10:00** at **Room temperature** ?

This step allows the gel pieces to subsequently imbibe iodoacetamide (Step 21).

21  30m

Alkylate peptides by addition of **20 millimolar (mM)** iodoacetamide in **40 millimolar (mM)** NH_4HCO_3 and incubate at **Room temperature** for **00:30:00** , **1200 rpm** .

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Samples should be kept in the dark during this step as iodoacetamide is light-sensitive.

22  10m

Dehydrate gel pieces by washing in 100% (v/v) ACN for **00:10:00** .

Perform this step on thermomixer set to **1200 rpm** at **Room temperature** . Repeat step 22 twice until the gel pieces appear completely dry and white.

23 

Remove supernatant using a pipette and vacuum dry gel pieces to remove any residual CAN.

24  10m

Add **100 ng** of protease in **100 µL** of appropriate buffer (See Table 1) to the gel pieces from step 23 and incubate **Overnight** on thermomixer at **37 °C** , **1200 rpm** .

Table 1 describes the different protease combinations used for total protein digestion and the appropriate buffers for each protease.

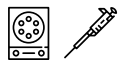
A	B
Protease	Buffer
Trypsin + LysC	50 mM TEABC
Asp-N	50 mM Tris-HCl
Chymotrypsin	100 mM Tris-HCl + 10 mM CaCl2

Table 1: Protease combinations used for total protein digestion and appropriate buffers for each protease.

Peptide extraction

25  10m

Supplement samples from step 24 with **50 µL** of extraction buffer (80% ACN in 0.2% Formic Acid) and incubate on thermomixer at **Room temperature** for **00:10:00** at **1200 rpm** .

26  1m

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Centrifuge samples for **00:01:00** at **2000 x g** to pellet the gel pieces and using a pipette carefully transfer the supernatant to a new low-binding? tube.

Ensure that the gel pieces are not transferred to the new tube when pipetting the supernatant.

27 Repeat step 25 until the gel pieces appear completely dried. Each time, transfer the supernatant into the same tube (from step 26).

28 Vacuum dry the combined supernatants (containing the digested peptides) and proceed with C18 clean-up protocol (as described in **C18 stage-tip protocol** section).

C18 stage-tip protocol:

29 

This protocol has been adapted from dx.doi.org/10.17504/protocols.io.bs3tngnn

Prepare single layer of C18 stage-tip using 16-gauge syringe needle[FT(1].

Prepare a single layer with 16-gauge needle and pass it with spray duster into the **250 µL** tip for **0.1 µg** to **5 µg** of peptide amount.

30 

Resuspend the vacuum dried peptides from step 28 in **80 µL** of Solvent A1 (0.1% (by vol) TFA in MQ-H2O).

31  2m

Add **80 µL** of 100% (by vol) ACN to the C18 stage-tip from Step 29 and centrifuge at **2000 x g** for **00:02:00** at **Room temperature** . Discard flow through.

This step is required to activate the C18 resin.

32 2m

Add **80 µL** Solvent A1 (0.1% (by vol) TFA (by vol) in MQ-H2O)) and centrifuge at **2000 x g** for **00:02:00** at **Room temperature** . Discard flow through. Repeat this step.

This step is required to equilibrate the C18 resin.

33 5m

Load the acidified peptide digest from Step 30 to the C18 stage-tip from step 32 and centrifuge at **1500 x g** for **00:05:00** at **Room temperature** .

During this step the peptides will absorb to the C18 resin.

34 5m

Reapply the flow through to the C18 stage-tip column and centrifuge at **1500 x g** for **00:05:00** at **Room temperature** .

35 2m

Add **80 µL** of Solvent A1 (0.1% (by vol) TFA v/v) in MQ-H2O)?) to the C18 stage-tip column and centrifuge at **2000 x g** for **00:02:00** at **Room temperature** . Discard flow through. Repeat again.

36 Place the C18 stage-tip from step 35 into a new 1.5 ml low binding tube.

Using new tubes is important to avoid contamination.

37 2m

Elute peptides from the C18 stage-tip by adding **40 µL** of Elution buffer (Solvent B1: 40% (by vol)

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acetonitrile in 0.1% (by vol) TFA) in MQ-H2O and centrifuge at **1500 x g** for **00:02:00** .

38 2m

Repeat step 37.
Elute peptides from the C18 stage-tip by adding **40 µL** of Elution buffer (Solvent B1: 40% (by vol) acetonitrile in 0.1% (by vol) TFA) in MQ-H2O and centrifuge at **1500 x g** for **00:02:00** .

39 Immediately snap freeze the eluted peptides from step 38 on dry ice and vacuum dry.

40 Perform mass spectrometry analysis of the peptides as described in **LC-MS/MS analysis** section.

LC-MS/MS analysis

41

Dissolve the peptides in LC-Buffer (3% ACN (v/v) in 0.1% Formic acid (v/v)).

Just **200 ng** of peptide digest per sample is good enough to achieve the coverage on Exploris 240 mass spectrometer. If the starting material of LRRK1 that was used for the Kinase assay is **1 µg** then split the sample into five aliquots of **200 ng** each to inject on MS. The reminder of the sample can be injected on a different mass spectrometer to get an alternative fragmentation to HCD such as EThCD on Lumos or EAD on Sciex Zeno-TOF 7600 MS platforms.

42

Take **200 ng** of the peptide digest of LRRK2 in **5 µL** or **10 µL** in LC-buffer and prepare it for the Evotips loading. The Evo tips are a versatile disposable trap columns that enables <0.1% carry-over between samples.

43 Prepare the Evotips as described in the Protocol in PMID: 33367571.

44 Place the Evotips on EvoSep autosampler and used the 30 sample per day (30SPD) method to execute the

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LC method through Xcalibur interface that is inline with Orbitrap Exploris 240 mass spectrometer.

45 EvoSep LC system injects and executes a partial elution of the sample from Evotip and loads onto the long storage loop in which the pre-formed gradient generated at the initial step. Following the loading the High-pressure pump pushes the sample into the analytical column (ReproSil-Pur C18, 1.9 µm beads by Dr Maisch. #EV1113).

46 The following MS instrument method can be constructed for the High-resolution HCD fragmentation analysis:

A	B	C
Instrument	Thermo Scientific Orbitrap Exploris 240	
LC system	EvoSep Liquid Chromatography system	30 SPD method
Method duration	45 min	
MS Global settings:		
	Infusion mode:	Liquid Chromatography
	Expected LC peak width (s):	15
	Advanced Peak determination:	TRUE
	Default charge state:	2
	Internal mass calibration:	off
Full scan settings:		
	Orbitrap resolution:	120000
	Scan range (m/z):	375-1500
	RF lens(%):	70
	AGC target:	Custom
	Normalized AGC target (%):	300
	Maximum injection Time mode:	Custom
	Maximum injection Time (ms):	25
	Micorscans:	1
	Data type:	Profile
	Polarity:	Positive
Filters:		
MIPS	Monoisotopic peak determination:	Peptide
	Relax restrictions when too few precursors are found:	TRUE
Intensity	Filter Type:	Intensity Threshold
	Intensity Threshold:	5.00E+03
Charge State	Include charge state(s):	2 to 6
	Include undetermined charge states:	False
Dynamic Exclusion	Dynamic Exclusion Mode:	Custom
	Exclude after n times:	1
	Exclusion duration (s):	5
	Mass Tolerance:	ppm

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	Low:	10
	High	10
	Exclude isotopes:	TRUE
	Perform dependent scan on single charge state per precursor only:	FALSE
Data Dependent	Data Dependent Mode:	Number of Scans
	Number of Dependent Scans	10
ddMS2 settings	Isolation Window (m/z):	1.2
	Isolation Offset:	Off
	Collision Energy Mode:	Fixed
	Collision Energy Type:	Normalized
	HCD Collision Energy (%):	28
	Orbitrap resolution:	15000
	First Mass (m/z):	110
	Scan range mode:	Auto
	AGC target:	Standard
	Maximum injection Time mode:	Custom
	Maximum injection Time (ms):	100
	Micorscans:	1
	Data type:	Profile
	Polarity:	Positive

Data analysis

47

Transfer the raw data to search with Thermo Scientific Proteome Discoverer 2.4 Software suite that is integrated with Sequest-HT search algorithm.

As the PD 2.4 software is commercial software suite, if you don't have access to it consider in using Open-source package like MaxQuant or FragPipe.

48 We recommend creating a custom protein sequence FASTA file rather than using the entire Uniprot Human or Mouse proteome FASTA file. For example: Copy the Human LRRK1 FASTA sequence and past it into a Notepad++ and save with LRRK1.FASTA .

Ensure if you have any N-ter or C-ter GFP or HA tag of a recombinant LRRK1 and append the sequence accordingly).

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49 Import the LRRK1.FASTA sequence into the PD 2.4 software.

50 Construct the Processing and Consensus workflows

A	B	C

The Processing workflow tree		
----------		
(0) Spectrum Files		
(1) Spectrum Selector		
(2) Sequest HT		
(3) Fixed Value PSM Validator		
(4) IMP-ptmRS		
(5) Minora Feature Detector		

Processing node 0	Spectrum Files	

Input Data		Note
File Name(s)		Specify the sample condtion and the Enyzme associated with the digestion
	RN-AM_211216_LRRK1_+PKC_Tryp-LysC_01.raw	
	RN-AM_211216_LRRK1_+PKC_Tryp-LysC_01.raw	
	RN-AM_211216_LRRK1_-PKC_Tryp-LysC_01.raw	
	RN-AM_211216_LRRK1_-PKC_Tryp-LysC_01.raw	

Processing node 1	Spectrum Selector	
----------		
1. General Settings		
Precursor Selection	Use MS1 Precursor	

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Use Isotope Pattern in Precursor Reevaluation	True	
Provide Profile Spectra	Automatic	
2. Spectrum Properties Filter		
Lower RT Limit	0	
Upper RT Limit	0	
First Scan	0	
Last Scan	0	
Lowest Charge State	0	
Highest Charge State	0	
Min. Precursor Mass	350 Da	
Max. Precursor Mass	5000 Da	
Total Intensity Threshold	0	
Minimum Peak Count	1	
3. Scan Event Filters		
Mass Analyzer	Is FTMS	
MS Order	Is MS2; MS1	
Activation Type	Is HCD	
Min. Collision Energy	0	
Max. Collision Energy	1000	
Scan Type	Is Full	
Polarity Mode	Is +	
4. Peak Filters		
- S/N Threshold (FT-only)	1.5	
5. Replacements for Unrecognized Properties		
Unrecognized Charge Replacements	Automatic	
Unrecognized Mass Analyzer Replacements	FTMS	
Unrecognized MS Order Replacements	MS2	
Unrecognized Activation Type Replacements	HCD	
Unrecognized Polarity Replacements	+	
Unrecognized MS Resolution@200 Replacements	120000	
Unrecognized MSn Resolution@200 Replacements	30000	
6. Precursor Pattern Extraction		

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Precursor Clipping Range Before	2.5 Da	
	5.5 Da	

Processing node 2	Sequest HT	
----------		
1. Input Data		
Protein Database	LRRK1.FASTA	
Enzyme Name	Trypsin (Full)	Here, specify AspN and Chymotrypsin separately fof the searches associated with those conditions
Max. Missed Cleavage Sites	2	
Min. Peptide Length	7	
Max. Peptide Length	144	
Max. Number of Peptides Reported	10	
2. Tolerances		
Precursor Mass Tolerance	10 ppm	
Fragment Mass Tolerance	0.05 Da	
Use Average Precursor Mass	False	
Use Average Fragment Mass	False	
3. Spectrum Matching		
Use Neutral Loss a Ions	True	
Use Neutral Loss b Ions	True	
Use Neutral Loss y Ions	True	
Use Flanking Ions	True	
Weight of a Ions	0	
Weight of b Ions	1	
- Weight of c Ions	0	
Weight of x Ions	0	
Weight of y Ions	1	
Weight of z Ions	0	
4. Dynamic Modifications		
Max. Equal Modifications Per Peptide	3	
Max. Dynamic Modifications Per Peptide	4	
- 1. Dynamic Modification	Oxidation / +15.995 Da (M)	
- 2. Dynamic Modification	Phospho / +79.966 Da (S, T, Y)	

Citation: Asad Malik, Raja Sekhar Nirujogi, Toan K. Phung, Dario R. Alessi Identification of PKC-regulated phosphosites on LRRK1 by mass spectrometry analysis https://dx.doi.org/10.17504/protocols.io.261gen89dg47/v1

7. Static Modifications		
- 1. Static Modification	Carbamidomethyl / +57.021 Da (C)	

Processing node 3	Fixed Value PSM Validator	
----------		
1. Input Data		
Maximum Delta Cn	0.05	
Maximum Rank	0	

Processing node 4	IMP-ptmRS	
----------		
1. Scoring		
PhosphoRS Mode	True	
Report only PTMs	True	
Use Diagnostic Ions	True	
Use Fragment Mass Tolerance of Search Node	True	
Fragment Mass Tolerance	0.5 Da	
Consider Neutral Loss peaks for CID, HCD and EThcD	Automatic	
Maximum Peak Depth	8	
Use a Mass accuracy correction	False	
2. Performance		
Maximum Number of Position Isoforms	500	
Maximum PTMs Per Peptide	10	

Processing node 5	Minora Feature Detector	
----------		
1. Peak & Feature Detection		
Min. Trace Length	5	
- Max. ΔRT of Isotope Pattern Multiplets [min]	0.2	
2. Feature to ID Linking		
PSM Confidence At Least	High	

Citation: Asad Malik, Raja Sekhar Nirujogi, Toan K. Phung, Dario R. Alessi Identification of PKC-regulated phosphosites on LRRK1 by mass spectrometry analysis https://dx.doi.org/10.17504/protocols.io.261gen89dg47/v1

A	B
The Consensus workflow tree	
----------	
(0) MSF Files	
(1) PSM Grouper	
(2) Peptide Validator	
(3) Peptide and Protein Filter	
(4) Protein Scorer	
(5) Protein Grouping	
(6) Peptide in Protein Annotation	
(15) Modification Sites	
(7) Protein FDR Validator	
(16) Peptide Isoform Grouper	
(10) Feature Mapper	
(11) Precursor Ions Quantifier	
Post-processing nodes	
----------	
(12) Result Statistics	
(13) Display Settings	
(14) Data Distributions	

Processing node 0	MSF Files
----------	
1. Storage Settings	
Spectra to Store	Identified or Quantified
Feature Traces to Store	All
2. Merging of Identified Peptide and Proteins	
Merge Mode	Globally by Search Engine Type
3. FASTA Title Line Display	
Reported FASTA Title Lines	Best match
Title Line Rule	standard
4. PSM Filters	
Maximum Delta Cn	0.05
Maximum Rank	0
Maximum Delta Mass	0 ppm

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Hidden Parameters	
MSF File(s)	RN-AM_211216_LRRK1_Sequest-Trypsin-(1).msf

Processing node 1	PSM Grouper
----------	
1. Peptide Group Modifications	
Site Probability Threshold	75

Processing node 2	Peptide Validator
----------	
1. General Validation Settings	
Validation Mode	Automatic (Control peptide level error rate if possible)
Target FDR (Strict) for PSMs	0.01
Target FDR (Relaxed) for PSMs	0.05
Target FDR (Strict) for Peptides	0.01
Target FDR (Relaxed) for Peptides	0.05
2. Specific Validation Settings	
Validation Based on	q-Value
Target/Decoy Selection for PSM Level FDR Calculation Based on Score	Automatic
Reset Confidences for Nodes without Decoy Search (Fixed Score thresholds)	False
----------	
Processing node 3	Peptide and Protein Filter
----------	
1. Peptide Filters	
Peptide Confidence At Least	High
Keep Lower Confident PSMs	False
Minimum Peptide Length	7
Remove Peptides without Protein Reference	False
2. Protein Filters	
Minimum Number of Peptide Sequences	1
Count Only Rank 1 Peptides	False
Count Peptides only for Top Scored Protein	False

Processing node 4	Protein Scorer

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No parameters	

Processing node 5	Protein Grouping
----------	
1. Protein Grouping	
Apply Strict parsimony principle	True

Processing node 6	Peptide in Protein Annotation
----------	
1. Flanking Residues	
Annotate Flanking Residues of the Peptide	True
Number Flanking Residues in Connection Tables	1
2. Modifications in Peptide	
Protein Modifications Reported	Only for Master Proteins
3. Modifications in Protein	
Modification Sites Reported	All And Specific
Minimum PSM Confidence	High
Report only PTMs	True
4. Positions in Protein	
Protein Positions for Peptides	Only for Master Proteins

Processing node 15	Modification Sites
----------	
1. General	
Report only PTMs	True
only Master Proteins	True
Motif Radius	10

Processing node 7	Protein FDR Validator
----------	
1. Confidence Thresholds	
Target FDR (Strict)	0.01
Target FDR (Relaxed)	0.05

Processing node 16	Peptide Isoform Grouper

Citation: Asad Malik, Raja Sekhar Nirujogi, Toan K. Phung, Dario R. Alessi Identification of PKC-regulated phosphosites on LRRK1 by mass spectrometry analysis https://dx.doi.org/10.17504/protocols.io.261gen89dg47/v1

No parameters	

Processing node 10	Feature Mapper
----------	
1. Chromatographic Alignment	
Perform RT Alignment	True
- Maximum RT Shift [min]	10
Mass Tolerance	10 ppm
Parameter Tuning	Coarse
2. Feature Linking and Mapping	
RT Tolerance [min]	0
Mass Tolerance	0 ppm
Min. s/N Threshold	5

Processing node 11	Precursor Ions Quantifier
----------	
1. General Quantification Settings	
Peptides to Use	Unique + Razor
Consider Protein Groups for Peptide Uniqueness	True
Use Shared Quan Results	True
Reject Quan Results with Missing Channels	False
2. Precursor Quantification	
Precursor Abundance Based on	Intensity
Min. # Replicate Features [%]	0
3. Normalization and Scaling	
Normalization Mode	Total Peptide Amount
Scaling Mode	On All Average
4. Exclude Peptides from Protein Quantification	
for Normalization	Use All Peptides
for Protein Roll-Up	Use All Peptides
for Pairwise Ratios	Exclude Modified
5. Quan Rollup and Hypothesis Testing	
Protein Abundance Calculation	Summed Abundances
N for Top N	3
Protein Ratio Calculation	Pairwise Ratio Based

Citation: Asad Malik, Raja Sekhar Nirujogi, Toan K. Phung, Dario R. Alessi Identification of PKC-regulated phosphosites on LRRK1 by mass spectrometry analysis https://dx.doi.org/10.17504/protocols.io.261gen89dg47/v1

Maximum Allowed Fold Change	100
Imputation Mode	None
Hypothesis Test	t-test (Background Based)
6. Quan Ratio Distributions	
- 1st Fold Change Threshold	2
- 2nd Fold Change Threshold	4
- 3rd Fold Change Threshold	6
- 4th Fold Change Threshold	8
- 5th Fold Change Threshold	10

51 If the database search is to be done using MaxQuant then refer below settings

A	B
Parameter	Value
Version	2.0.3.0
User name	RNirujogi
Machine name	MRC-MS-R640-4
Date of writing	05/23/2022 15:15:41
Include contaminants	TRUE
PSM FDR	0.01
SM 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	2
Use delta score	FALSE
iBAQ	FALSE
iBAQ log fit	FALSE

Citation: Asad Malik, Raja Sekhar Nirujogi, Toan K. Phung, Dario R. Alessi Identification of PKC-regulated phosphosites on LRRK1 by mass spectrometry analysis https://dx.doi.org/10.17504/protocols.io.261gen89dg47/v1

Match between runs	FALSE
Find dependent peptides	FALSE
Fasta file	C:\Raja\Database\LRRK1.FASTA
Decoy mode	revert
Include contaminants	TRUE
Advanced ratios	TRUE
Fixed andromeda index folder	
Combined folder location	
Second peptides	TRUE
Stabilize large LFQ ratios	TRUE
Separate LFQ in parameter groups	FALSE
Require MS/MS for LFQ comparisons	TRUE
Calculate peak properties	FALSE
Main search max. combinations	200
Advanced site intensities	TRUE
Write msScans table	FALSE
Write msmsScans table	TRUE
Write ms3Scans table	TRUE
Write allPeptides table	TRUE
Write mzRange table	TRUE
Write DIA fragments table	FALSE
Write DIA fragments quant table	FALSE
Write pasefMsmsScans table	TRUE
Write accumulatedMsmsScans 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

Citation: Asad Malik, Raja Sekhar Nirujogi, Toan K. Phung, Dario R. Alessi Identification of PKC-regulated phosphosites on LRRK1 by mass spectrometry analysis https://dx.doi.org/10.17504/protocols.io.261gen89dg47/v1

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)	20 ppm
Top MS/MS peaks per Da interval. (Unknown)	12
Da interval. (Unknown)	100
MS/MS deisotoping (Unknown)	TRUE
MS/MS deisotoping tolerance (Unknown)	7
MS/MS deisotoping tolerance unit (Unknown)	ppm
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 (ST)Sites.txt

Data analysis and Visualization

52 Manually verify the MS/MS spectrum and phosphorylation localization score within PD2.4.

53 Now export the filtered Phosphosites from modifications table for each of the sample/category

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Use the below scripts for parsing and combining the data to generate a heatmap representation.

The below script can also be accessed from the Alessi lab gihub web page: https://github.com/Alessi-Lab/LRRK1_phosphosites)

The script below would first read phosphosite mapping result, then map them on to the original protein amino acid sequence through combining PeptideGroups and ModificationSites result text file. The data would be filtered by probability greater or equal to 75 and grouped by the different tryptic digestion enzymes used. Only entries with the highest abundance values according to the unique motif, position and sample condition are kept. Then based on the sequence length, the data was divided into instances of 500 amino acid continuous span on the protein sequence. Each of these instances would be used to create a heatmap where the abundance of the peptide would be the heatmap color, the sample condition would be presented on the X-axis while the position of the phosphosites are represented in the Y-axis in ascending order.

```
import numpy as np
import pandas as pd
from glob import glob
import re
import seaborn as sns
import matplotlib.pylab as plt
if __name__ == "__main__":
proteases = ["AspN", "Chymotrypsin",
#"Trypsin"
]
files = ["PeptideGroups", "ModificationSites"]
phospho_re = re.compile(r"Phospho [S(\d+)\((\d+)\)]")
results = {}
for i in glob(r"\\mrc-smb.lifesci.dundee.ac.uk\mrc-group-
folder\ALESSI\Toan\TS22D4_Phosphosite mapping_02\*.txt"):
for p in proteases:
if p in i:
for f in files:
if f in i:
if p not in results:
results[p] = {}
results[p][f] = pd.read_csv(i, sep="\t")
break
break
merged_df = []
columns = set()
for p in proteases:
pg = results[p][files[0]]
ms = results[p][files[1]]
for i, r in pg.iterrows():
pg.at[i, "Primary IDs"] = ";".join([r["Master Protein Accessions"],
r["Annotated Sequence"][4:len(r["Annotated Sequence"])-4]])
```


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```
phos = []
s = re.search("\[(\d+)-(\d+)\]", r["Positions in Master Proteins"])

pos = []
if s:
pg.at[i, "Start"] = s.group(1)
mod_count = r["Modifications"].count("]; ")
if mod_count > 0:
for m in r["Modifications"].split("]; "):
if "Phospho" in m:
s = re.search("\[(.+)", m)
if s:
for si in s.group(1).split("; "):
sire = re.search("(\w)(\d+)\(", si)
if sire:
phos.append("".join([sire.group(1), sire.group(2)]))
pos.append(str(int(sire.group(2)) + int(pg.at[i, "Start"]) - 1))
else:
if "Phospho" in r["Modifications"]:
s = re.search("\[(.+)", r["Modifications"])
if s:
for si in s.group(1).split("; "):
sire = re.search("(\w)(\d+)\(", si)
if sire:
phos.append("".join([sire.group(1), sire.group(2)]))
pos.append(str(int(sire.group(2)) + int(pg.at[i, "Start"]) - 1))
pg.at[i, "Position"] = pos
pg.at[i, "Phospho"] = phos

pg = pg.explode(["Phospho", "Position"])
pg = pg[pd.notnull(pg["Phospho"])]
pg["Position"] = pg["Position"].astype(int)
for i, r in ms.iterrows():
ms.at[i, "Primary IDs"] = ";".join([r["Protein Accession"], r["Peptide
Sequence"]])
rpg = pg[[i for i in pg.columns if i.startswith("Abundance")] + ["Primary
IDs", "Phospho", "Position", "Modifications"]]
rename = {}
for i in rpg.columns:
if "Abundance" in i:
rename[i] = re.sub("Abundance: F\d+: Sample, ", "", i)
columns.add(rename[i])
print(rpg["Primary IDs"])
print(ms["Primary IDs"])
rpg = rpg.rename(columns=rename)
ms["Phospho"] = ms["Target Amino Acid"] + ms["Position in
Peptide"].astype(str)
ms["Enzymes"] = p
df = ms.merge(rpg, left_on=["Primary IDs", "Phospho"], right_on=["Primary
IDs", "Phospho"])
merged_df.append(df)
```


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```
merged_df = pd.concat(merged_df, ignore_index=True)
merged_df = merged_df[merged_df["Site Probability"]>=75]
result = pd.melt(merged_df, id_vars=[
"Phospho", "Position_y", "Enzymes", "Motif"], value_vars=list(columns),
var_name="Samples", value_name="Abundance")

a = result.groupby([
#"Phospho",
"Position_y", "Samples", "Enzymes", "Motif"]).max()

a.reset_index(inplace=True)
print(a["Samples"])
a["Conditions"], a["Replicates"] = a["Samples"].str.split("Rep-",
expand=True)
for i, g in a.groupby([
# "Phospho",
"Position_y", "Motif"]):
remove_motif = True
for i2, g2 in g.groupby(["Enzymes", "Conditions"]):
if len(g2[pd.notnull(g2["Abundance"])].index) > 1:
remove_motif = False
break
if remove_motif:
a["Motif"].loc[g.index] = ""

a.sort_values("Position_y", inplace=True)
e = 1
n = 500
samples = a["Samples"].unique()
samples_columns = []
for p in proteases:
for s in samples:
samples_columns.append((p, s))
multiindex = pd.MultiIndex.from_tuples(samples_columns, names=["Enzymes",
"Samples"])
while n:

c = a[(a["Position_y"] <= n)&(a["Position_y"] > (n-500))]
fontsize_pt = plt.rcParams['ytick.labelsize']
dpi = 72.27
top_margin = 0.2
bottom_margin = 0.2
left_margin = 0.2
right_margin = 0.2
figure_height = (len(c.index)/10) / (1 - top_margin - bottom_margin)
figure_width = 10 / (1-left_margin-right_margin)
c = c.set_index([
#"Phospho",
"Position_y", "Samples", "Enzymes", "Motif"])
c = c.unstack("Enzymes")

b = pd.pivot_table(c, values="Abundance", columns="Samples", index=
```


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```
["Position_y",
#"Phospho",
"Motif"])
b.fillna(0, inplace=True)
b = b.T

for i in b.columns:
b0 = b[i][b[i]==0]
b[i] = (np.log2(b[i], where=b[i]>0) - np.log2(b[i], where=b[i]>0).mean()) /
np.log2(b[i], where=b[i]>0).std(ddof=1)
for ind in b0.index:
b[i].loc[ind] = np.nan
b = b.T
new_df = pd.DataFrame(index=b.index, columns=multiindex)
for i in new_df.columns:
if i in b.columns:
new_df[i] = b[i]
else:
new_df[i].fillna(0, inplace=True)

new_df.to_csv(f"merged{n}.csv")
fig, ax = plt.subplots(
figsize=(figure_width, figure_height),
gridspec_kw=dict(top=1-top_margin, bottom=bottom_margin, left=left_margin,
right=1-right_margin)
)
mask = np.isnan(b)
sns.heatmap(new_df, cmap="YlGnBu", mask=mask, square=True, ax=ax)
ax.set_facecolor("silver")
ax.xaxis.tick_top()
ax.xaxis.set_label_position('top')
for label in ax.get_yticklabels():
label.set_weight("bold")
for label in ax.get_xticklabels():
label.set_weight("bold")
plt.xticks(rotation=90)
plt.savefig(f"result{n}.pdf")
for i, r in b.iterrows():
if i[1] != "":
p = re.compile(r"[RK]\w[ts]\w\w[RK]")
s = re.search(p, i[1])
if s:
print(i)
n += 500
e += 1
if n >= a["Position_y"].max():
break
```


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