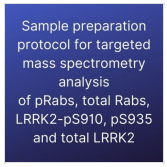


Version 1

May 06, 2021

Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2 V.1

Raja S. Nirujogi[1], Dario Alessi[1]

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

1 Works for me dx.doi.org/10.17504/protocols.io.busynwfw

Dario Alessi

ABSTRACT

Leucine rich repeat kinase2 (LRRK2) is one of the most commonly implicated kinase and mutations that hyperactivate kinase activity is seen in familial Parkinson's disease. LRRK2 phosphorylates a sub-set of small Rab GTPase proteins within their Switch-II motif. We recently described a multiplexed targeted mass spectrometry assay to identify and quantify both phosphorylated Rab proteins and well known LRRK2 phosphorylation sites such as LRRK2-pS910/pS935(PMID: 33367571). Here we describe a detailed protocol for the sample preparation of cells/tissues that are derived from LRRK2 mutation knock-in models. We outline a simple Triton-X 100 or SDS lysis that are straightforward to capture solubilized proteins amenable for both immunoblotting and mass spectrometry analysis. A stepwise protocol to buffer exchange Triton-X 100/SDS using S-Trap columns and on-column trypsin digestion. We also describe an optimized condition to conjugate required antibodies for the selective enrichment of pRabs and LRRK2 phosphopeptides and total LRRK2 peptide including the protocol for peptide immunoprecipitation and subsequent Stage tip-based desalting of the samples prior to targeted mass spectrometry analysis. We provide a cautionary step's in each sections of the protocol for a reproducible sample preparation. We believe this protocol is an easy handout to study LRRK2 activity both from cells/tissues including samples from human neutrophils.

ATTACHMENTS

Sample preparation protocol for targeted MS analysis of pRabs and LRRK2.pdf

DOI

dx.doi.org/10.17504/protocols.io.busynwfw

PROTOCOL CITATION

Raja S. Nirujogi, Dario Alessi 2021. Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. **protocols.io** https://dx.doi.org/10.17504/protocols.io.busynwfw Version created by Dario Alessi

KEYWORDS

Mass Spectrometry, Mass Spec, pRabs, total Rabs, LRRK2-pS910, pS935, total LRRK2

LICENSE

This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

CREATED

May 06, 2021

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

LAST MODIFIED

May 06, 2021

OWNERSHIP HISTORY

May 06, 2021 Dario Alessi

PROTOCOL INTEGER ID

49720

GUIDELINES

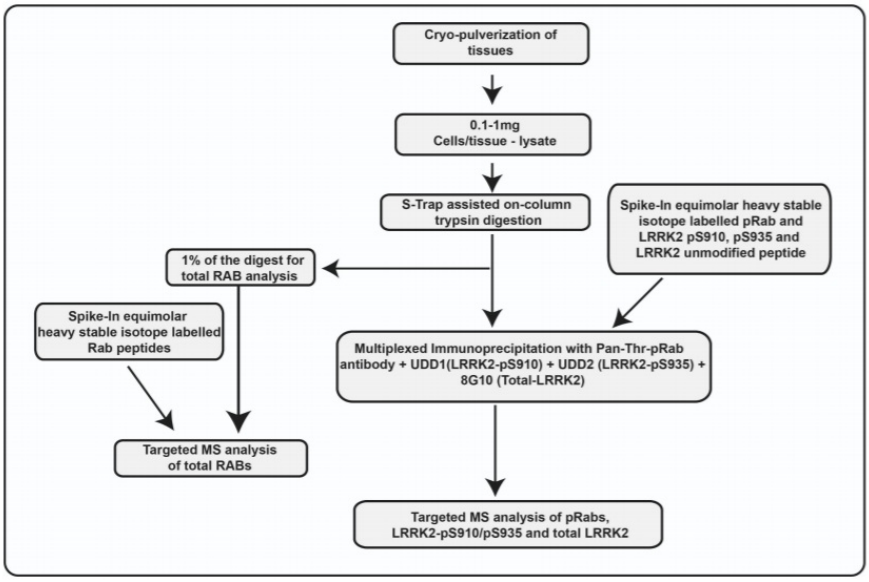


Fig. 1) Workflow depicting the sample preparation for pRab's and LRRK2 pS910/pS935, total LRRK2 targeted mass spectrometry analysis

MATERIALS TEXT

Consumables:

1) 1.5 ml protein low bind Eppendorf tubes (Eppendorf™ #022431081)
2) Marker pen
3) Pipette set (1 ml, 200 µl, 100 µl, 20 µl, 10 µl)
4) Pipette tips low binding (1 ml, 250 µl, 10 µl, Star labs Bevelled tips refill # S1111-3700, S1111-1706, S1111-6700)
5) PPE kit (Lab coat, gloves, safety glasses)
6) Dry ice
7) Liquid Nitrogen
8) Ice bucket
9) 1.5 ml eppendorf tubes rack
10) 96 well plate- clear (Geneier Bio-one #655101)
11) 2 ml tubes (Axygen™ MCT200O)
12) 16-gauge needle (# Z261378. Sigma Aldrich)
13) Spray duster (Qconnect #KFO4499)
14) X100 20 mL Amber Glass EPA Vial (Thermo Scientific™ EPA Screw Vial Assembled Kit, 20 mL amber glass EPA vial with cap and seal. Fisher Scientific # 11543750)
15) X72 40 mL Amber class EPA vial W Cap and seal (Thermo Scientific™ EPA, TOC, and Scintillation Vials & Closures. Fisher Scientific # 12418656) (Note: Prepare all stock and working reagents in these amber vials to store as per the protocol)
16) Millipore pH Strips (VWR # 1.09584.0001)
17) BCA protein assay kit (Pierce # 23225)
18) PTFE- O rings (To place the stage-tip into the Eppendorf tubes. Generally, you could get from NEST group desalting columns and re use them https://www.nestgrp.com/)
19) S-Trap mini columns ((https://www.protifi.com/))

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

20) Sep-Pak Vac 1cc (50 mg) tC18-Cartridges (Waters # WAT054960)
21) Evotips (EvoSep #EV2001)
22) EvoSep 8cm analytical column (EvoSep #EV-1064)
23) EvoSep 15cm performance analytical column (EvoSep #EV-1118)
24) Easy-Spray PepMap RSLC C18 2um, 50cmx75um (Thermo Fisher Scientific #ES802)
25) Heavy stable synthetic peptides. Custom design by JPT peptide technologies (https://www.jpt.com/)
26) Pierce retention time calibration mix (Thermo Fisher Scientific #88321)

Reagents:
1) SDS Lysis Buffer: Final 2% (by mass) SDS in 100 mM Triethylammonium bicarbonate pH 8.5 (TEABC, this is the natural pH of this buffer and made from a 1 M TEABC stock purchased from Sigma Catalogue number# T7408-500 mL), 1 mM sodium orthovanadate, 50 mM NaF, 10 mM b- glycerophosphate, 5 mM sodium pyrophosphate, 1 µg/ml microcystin-LR, and complete EDTA-free protease inhibitor cocktail (Roche)
2) 1% Triton-x 100 Lysis buffer: Final 1% (by mass) SDS in 100 mM Triethylammonium bicarbonate pH 8.5 (TEABC, this is the natural pH of this buffer and made from a 1 M TEABC stock purchased from Sigma Catalogue number# T7408-500 mL), 1 mM sodium orthovanadate, 50 mM NaF, 10 mM b- glycerophosphate, 5 mM sodium pyrophosphate, 1 µg/ml microcystin-LR, and complete EDTA-free protease inhibitor cocktail (Roche)
3) Tris (2-carboxyethyl) phosphine (TCEP) (Sigma Aldrich # 75259-10G). (Note: Prepare and store 10 µl aliquots of 1 M TCEP in Milli-Q H2O. Prior to use dilute the 1 m TCEP solution 10 x in 300 mM TEABC to generate a stock solution of 0.1 M TCEP in 300 mM TEABC).
4) Ortho-Phosphoric acid 85% (by vol) (Sigma #5438280100). (Note: Prepare 12% (by vol) stock aqueous phosphoric acid by diluting in water and store in 4oC)
5) S-Trap protein binding buffer (90% (by vol) aqueous LC grade methanol containing a final concentration of 100 mM TEAB, pH 7.1, made from a 1 M TEABC stock purchased from Sigma Aldrich # T7408-500 mL)
6) Trypsin from bovine pancreas TPCK treated (Sigma Aldrich #V5111). (Note: Prepare a stock at 5 µg/µl of 50 µl volume vials and store in -20 freezer)
7) CBQCA Protein Quantitation Kit (Thermo Fisher Scientific #C-667)
8) 10X Immunoaffinity purification (IAP) buffer. Prepare 10X IAP buffer containing 500mM MOPS, 100 mM Na2HPO4 and 500 mM NaCl as a stock and dilute it with Milli-Q H2O to achieve 1X as working solution and store at 4oC (MOPS 3-(N-Morpholino) propanesulfonic acid, 4-Morpholinepropanesulfonic acid (Sigma Aldrich # 69947-500G; Sodium phosphate dibasic Na2HPO4 dibasic (Sigma Aldrich # S7907-500G); Sodium chloride (Sigma Aldrich # S7653-1KG)
9) Gibco™ PBS, pH 7.4 (Fisher Scientific # 11593377)
10) Protein A/G beads (https://mrcppureagents.dundee.ac.uk/)
11) Pan-pRab8 antibody (Abcam #MJF-R20, ab230260)
12) LRRK2 pS910 antibody (UDD1 https://mrcppureagents.dundee.ac.uk/)
13) LRRK2 pS935 antibody (UDD2 https://mrcppureagents.dundee.ac.uk/)
14) LRRK2 8G10 antibody (8G10 https://mrcppureagents.dundee.ac.uk/)
15) Methanol (VWR # 1.06035.2500)
16) Acetic acid 100% (Sigma#5438080100)
17) LC-MS grade Acetonitrile (VWR # 1.00030.2500)
18) Iodoacetamide (Sigma # I1149)
19) LC grade Formic acid (Sigma # 695076)
20) Trifluroacetic acid (Sigma # 302031) (Note: Prepare and store 20% (by vol) aqueous TFA stock at 4oC)
21) Empore C18 disks, 47 mm (CDS analytical #2215) (Note: Prepare a single layer with 16-gauge needle and pass it with spray duster into the 250 µl tip for 0.1 to 5 ug of peptide amount. For more than 5 ug use 2 or 3 layers of C18 material. Refer Figure 1 -see below for Stage-tip assembly).
22) 20% (by mass) aqueous SDS stock
23) LC buffer (0.1% (by vol) Formic acid in 3% (by vol) Acetonitrile)
24) Solvent-A1 (0.1% (by vol) TFA)
25) Solvent-A2 (0.1% (by vol) Formic acid
26) Solvent-B1 (50% (by vol) acetonitrile 0.1% (by vol) TFA)
27) Solvent-B2 (60% (by vol) acetonitrile 0.1% (by vol) Formic acid)

Equipment:
1) Pulveriser kit (https://cellcrusher.com/)
2) -80°C deep freezer, -20°C freezer and 4°C fridge
3) Benchtop centrifuge (VWR)
4) Milli-Q water system
5) Orbital shaker
6) pH meter
7) Ice rack

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

8) Plate reader for Protein quantification (BioTek Epoch)
9) Diagenode Bioruptor plus sonication system
10) Eppendorf Thermomixer
11) Thermo Savant Speedvac system (#SPD140DDA)
12) 1.5 ml tube floaters
13) PHERAstar FSX
14) Branson water bath sonicator
15) Dionex RSLC 3000 nano-LC system
16) Dionex RSLC 3000 LC system for Offline fractionation with Auto sampler or Fraction collector, micro pump and VWD detector
17) Orbitrap Fusion Lumos Tribrid Mass spectrometer or Q Exactive HF-X or Orbitrap Exploris 480 or Orbitrap Exploris 240 mass spectrometers
18) Thermo Savant Speed vac system (#SPD140DDA)
19) Nanodrop 1000 (Thermo Fisher Scientific)
20) Rubber bulb # Fisher brand™ Rubber Pipette Bulb# 12446180

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Lysate preparation from mice tissues

1 Snap freeze mouse tissue immediately after isolation in liquid nitrogen and store at **-80 °C** .

2 Pulverize frozen tissue in liquid nitrogen to a fine powder and snap freeze immediately in 2ml tubes. This is done using cell crusher kit.

Maintain all Pulverizer units in liquid nitrogen including spatula. After use wash the pulveriser with a tap water and clean thoroughly several times with **70 % (v/v) ethanol** .

3 Weigh pulverized tissue powder on a weighing balance.

5-7% of the wet weight of tissue should correspond to a protein amount of ~ **5 mg** to **7 mg protein** / **100 mg wet weight** .

Keep samples on dry ice during and after weighing. Take a 1.5 ml tube check the weight on weighing balance and subsequently tare the weight. Now take small scoop of sample using pre-chilled fine spatula to weigh the sample

4 

Immediately add **10 X weight of the tissue** of **2 Mass Percent SDS lysis buffer** .

For e.g. **100 mg tissue powder** please add **1000 µl lysis buffer** .

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

5 Put samples on an orbital shaker in cold room at **1000 rpm, 00:15:00** .

6 Boil samples at **95 °C** for **00:10:00** and allow to cool. 10m

7 Sonicate samples using a Diagenode Bioruptor (at high energy for 10 cycles (**00:00:30 ON** / **00:00:30 OFF**)). 1m

8  30m

Clarify lysate by centrifuging at **20800 rpm, 4°C, 00:30:00** .

9 

Measure protein amount using the Bicinchoninic acid assay (BCA) method in triplicate at 1:10 and 1:20 dilutions-repeat analysis if readings are not close.

10 

Take **300 µg protein amount** for Mass spectrometry analysis.

In case of mice brain tissues the minimum recommended lysate amount is **1 mg** to identify and quantify endogenous pRab proteins and LRRK2 pS910/pS935 and total LRRK2 peptide.

Lysate preparation from cells 1h 30m

11 For cells such as A549 or HEK293 or Mouse embryonic fibroblasts (MEFs): Culture them in 6cm or 10cm dish to a ~70% confluency and complete the required treatments such as MLi2 treatment for about **01:30:00** . 1h 30m

12 

Harvest cells by washing with 1X plain DMEM media without any serum and aspirate the media completely.

13 

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Lyse cells using 1% Triton-X 100 lysis buffer.
Add **150 µl** to a 6cm dish or **350 µl** to a 10cm dish.

14 Scrape cells using cell scraper and collect the lysate using 1 ml non-autoclaved tips into a protein lo binding tubes.

15 

50m

Keep cells **On ice** for **00:30:00** and centrifuge at **18000 x g, 4°C, 00:20:00** .

16 

Estimate protein amounts using BCA assay.

17 Take **300 µg protein amount** for Mass spectrometry analysis (cell lysate or tissue lysate).

SDS-clean up and S-Trap assisted trypsin digestion

18 

Aliquot **300 µg protein amount** in 1.5 ml Protein lo-binding tubes.

19 

Perform reduction by adding 1:10 dilution of a solution of 0.1 M TCEP dissolved in 300 mM TEABC to bring final concentration to **10 Milimolar (mM) TCEP** .

20 

Incubate on a Thermomixer with a gentle agitation at **1000 rpm, 60°C, 00:30:00** .

21 

Bring tubes to **Room temperature** and add a 1:10 dilution of freshly prepared **0.4 Molarity (M) iodoacetamide** dissolved in water.

It is critical that the samples are at room temperature prior to addition of iodoacetamide.

22 

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Incubate in dark on a Thermomixer with a gentle agitation at **1000 rpm, Room temperature , 00:30:00** .

23

Quench alkylation by addition of a 1:10 dilution of a solution of 0.1 M TCEP dissolved in 300 mM TEABC to bring final concentration to **[M]10 Milimolar (mM) TCEP** .
If samples are not quenched after alkylation proceed immediately to the next steps below.

At this stage sample can be stored in **-80 °C** freezer.

24

Incubate on a Thermomixer with a gentle agitation at **1000 rpm, Room temperature , 00:20:00** .

25

Add SDS to a final concentration of **[M]5 Mass Percent** from **[M]20 Mass Percent SDS stock** .

The lysate is already in 2% (by mass) SDS, so supplement with a stock of 20% (by mass) SDS in order to bring the final SDS concentration to 5% (by mass). For lysates with 1% Triton-x 100, it is important to add final 5% (by mass) SDS to the samples to ensure complete denaturation and subsequent capturing of fine protein particles by S-Trap columns.

26

Add a 1:10 dilution of **[M]12 % volume phosphoric acid** into the sample to make a final concentration of ~ **[M]1.2 % volume phosphoric acid** .

Note for example, **5 µl** to **50 µl** .

27

Dilute the sample **7 times** in S-Trap wash buffer (**[M]90 % volume methanol** in **[M]0.1 Molarity (m) TEAB pH 7.1 v/v**).

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

For example, if sample volume is **50 µl** then add **300 µl S-Trap wash buffer** (**90 % volume methanol** in **0.1 Molarity (m) TEAB pH 7.1 v/v**).

If the lysate volume is more than 50 µl, scale S-Trap wash buffer accordingly i.e. 7 times the volume of the lysate

28 Prepare an S-Trap mini column in a 2 ml tube.

29 

Add the diluted protein mixture from step 27 to the column.

30  1m

Centrifuge briefly at **1000 x g, Room temperature , 00:01:00** to capture the protein particles.

All centrifugation steps should be at room temperature. If you observe any clogging then increase the centrifugation speed up to a maximum of 4,000g or increase the centrifugation time up to 2-4 minutes. . If the total volume of Lysate +S-Trap buffer mixture is >500 µl then add it to the column multiple times until the sample passes through the column and discard flow through

31 Wash column with **400 µl S-Trap buffer** for a total of 4 times (spin **1000 x g, 00:01:00** between washes): 1m

Note that the protein remains bound on the column and SDS and buffer components that effect trypsin digestion are removed.

31.1  1m

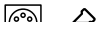
Wash column with **400 µl S-Trap buffer**

Spin **1000 x g, 00:01:00** (1/4)

31.2  1m

Wash column with **400 µl S-Trap buffer**

Spin **1000 x g, 00:01:00** (2/4)

 1m

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

31.3

Wash column with **400 µl S-Trap buffer**

Spin **1000 x g, 00:01:00** (3/4)

31.4 1m

Wash column with **400 µl S-Trap buffer**

Spin **1000 x g, 00:01:00** (4/4)

32 Move the S-Trap column to a clean 2 ml tube for digestion.

33

Add **160 µl freshly dissolved trypsin solution** containing **15 µg trypsin** dissolved in **100 Milimolar (mM) TEAB (1:20)** .

Prepare and store Trypsin stocks in Milli-Q water at **5 µg/µl** of 50µl vials and store in **-20 °C** freezer.

34 1m

Centrifuge briefly at **200 x g, 00:01:00** .

35 Collect flow through and reapply the trypsin solution back onto the column being careful to avoid air bubbles.

36 1h 30m

Cap the tubes and incubate at **47 °C** without shaking for **01:30:00** .

Do not shake as this causes bubbles and damages the column.

37 2h

Incubate for another **02:00:00** at **Room temperature** .

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

38

Add **80 µl 50 mM TEAB** then spin to elute.

Place the eluate in a new 1.5 ml Eppendorf tube termed “eluate tube”.

39

Next, add **80 µl 0.15% (by vol) Formic Acid** and spin to elute.

Also add this eluate to the “eluate tube”.

40

Finally, add **80 µl 50% (by vol) Acetonitrile in 0.15% (by vol) formic acid** and spin to elute.

Also add this eluate to the “eluate tube”.

3 eluates should have been added to the eluate tube.

41

Take **1 µl** - **2 µl combined eluate** , vacuum dry and inject on MS to verify the digestion efficiency.

Analyse data with a 70 min gradient run on QE HF-X or Orbitrap Lumos mass spectrometer in a F1-T-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%

42

Estimate peptide amounts using CBQCA assay kit and take equal amounts of peptide digest for each sample and Vacuum dry using SpeedVac.

Aliquot **5 µg peptide digest** separately for total Rab analysis.

Pan-pRab, UDD1, UDD2 and 8G10 antibodies conjugation to Protein A/G beads 3m

3m

43

Take required amount of Protein A/G beads and wash with a total of

1 mL slurry volume + reminder is PBS wash volume (1X PBS buffer) . Centrifuge at

2000 x g, 00:03:00 .

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

(e.g. For 24 samples with 2 rounds of immunoprecipitations **480 µl bead slurry** is required. **10 µl slurry** /sample for one round of immunoprecipitation).

Let the beads settle down for **00:02:00** before aspirating the supernatant.

44 Repeat wash steps with **1 mL 1X PBS** two more times:

44.1

Wash beads with **1 mL 1X PBS** . (1/2)

44.2

Wash beads with **1 mL 1X PBS** . (2/2)

45

Incubate an equimolar concentration of Pan-pRab, UDD1, UDD2 and 8G10 antibodies to give a concentration of **50 ng/ul** of slurry.

(Example, for 24 samples with two round of Immunoprecipitation, you require **24 µg Pan-pRab** + **24 µg UDD1** + **24 µg UDD2** + **24 µg 8G10** in a total of **480 µl protein A/G slurry** which will give 50ng/µl concentration).

Due to the high binding capacity of Protein A/G beads all four antibodies can be simultaneously conjugated with 25 µg can be multiplexed to conjugate with **500 µl Protein A/G beads slurry** .
For 24 samples 12 µg of each of the antibodies are required i.e. 0.5 µg per sample

46

Add extra 1X PBS to a final volume of **1 mL** .

47 3h

Incubate at **4 °C** for **03:00:00** on an end-to-end rotator.

48 2m

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Post incubation, centrifuge at **2000 x g, 4°C, 00:02:00** and collect the supernatant to be used as a flow-through fraction to confirm by SDS-PAGE that the antibodies are bound to the beads completely.

49

Wash with **1 mL 1X PBS** for a total of 3 times:

49.1

Wash with **1 mL 1X PBS** . (1/3)

49.2

Wash with **1 mL 1X PBS** . (2/3)

49.3

Wash with **1 mL 1X PBS** . (3/3)

50

Now add 1X PBS to a final volume of **480 µl** to achieve an antibody concentration of **50 ng/ul beads slurry** . This mixture is stable at **4 °C** for up to two months. For a longer storage add **0.03 % volume sodium azide** .

Preparation and storage of heavy/light stable synthetic peptides

51 Heavy/light synthetic peptides generally come in a lyophilized form at a net amount of **1 Nanomolar (nM)** .

Our lab has pRab1, pRab3, pRab8, pRab10, pRab35 and pRab43 and LRRK2 pS910 and pS935 and total LRRK2 heavy synthetic peptides.

52 Dissolve peptides in **100 µl reconstitution buffer** . Leave tubes **On ice** for about **00:20:00** and then sonicate using a water bath sonicator for **00:05:00** . 25m

Reconstitution buffer: **3 % volume Acetonitrile** in **0.1 % volume formic acid** in HPLC-grade water to achieve 10 pmol/µl.

53 30s

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Vortex and centrifuge tubes using benchtop microfuge for **00:00:30** .

54 Now prepare dilutions using reconstitution buffer as required (e.g. 10pmol/ µl, 1pmol/µl, 100 fmol/µl and 10fmol/µl.) From these stocks prepare an equimolar concentration of all six pRabs+3 LRRK2 peptides to get 1pmol/µl or 100fmol/µl or 10fmol/µl concentrations to be Spiked into the peptide mixture just before immunoprecipitation. Store these stocks in **-80 °C** freezer.

For example, to achieve 1000 fmol/µl of an equimolar concentration of 200 µl final volume, take 20 µl from each peptide (6 Rabs+3LRRK2 peptides) from a stock of 10 pmol/µl to a total volume of 160 µl and then add 40 µl of reconstitution buffer to achieve a final concentration of 1000fmol/µl in 200 µl volume.

55 Maintain 10 fmol/µl as working stocks so that **10 µl** should give **100 fmol** net amount in immunoprecipitation reaction.

To avoid pipetting errors it is recommended to maintain the desired amount that will be spiked into the peptide mixture to be at a minimum of 10µl. e.g. For 100 fmol add 10µl of 10 fmol/µl stock. If you intend to Spike 50 fmol amount, then add 10 µl of 5fmol/µl stock

Peptide immunoprecipitation 3m

56 12m

Spin the lyophilized peptides from Step 42, at **17000 x g, Room temperature , 00:02:00** to ensure everything settles down at the bottom of the tube. Add **1 mL 1X IAP buffer** , keep samples on water bath sonication for **00:10:00** . The pH should be around **pH 7.2** .

Use pH strips that have a fine gradient scale from pH 1.0 to pH 10, to adjust the pH

56.1 10m

If the digest is still acidic, adjust the pH by adding **5 µl 1 M Tris pH 12.0** . Centrifuge at high speed at **Room temperature** for **00:10:00** to remove any insoluble traces and transfer supernatant to new 1.5 ml Eppendorf tube.

57

Keep samples **On ice** . In the meantime, aliquot required amount of antibody slurry from the stock as prepared in Step 49.

For each peptide immunoprecipitation containing up to **100 µg** - **150 µg tryptic peptide** we use

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

10 µl slurry containing **0.5 µg Pan-pRab** + **0.5 µg UDD1** + **0.5 µg UDD2** + **0.5 µg 8G10** antibody. Also, add antibody cocktail using nonautoclaved cut tips.

Due to low abundance of pRabs+LRRK2 pS910, pS935 in brain the recommended peptide amount to be used is >600 µg peptide digest derived from 1 to 1.5mg starting lysate.

58

Add [M]**100 fmol** amount of an equimolar heavy stable peptides derived from step 55.

We use an equimolar mixture of 10µl of 10fmol/µl stock to achieve 100 fmol amount containing six pRabs and LRRK2 pS910, pS9135 and unmodified peptide

59 2h

Incubate the mixture at **4 °C** for **02:00:00** on an end-to-end rotator.

60 2m

After the incubation, centrifuge at **1500 x g, 4°C, 00:02:00** . Collect the flow through and transfer it to new protein lo-binding 1.5 ml Eppendorf tubes for second round of immunoprecipitation.

61 Keep beads on an ice-block **On ice** until the second round of immunoprecipitation is completed.

62 2h

To the flowthrough from the 1st round of immunoprecipitation (Step 60), add another **10 µl antibody slurry** containing **0.5 µg Pan-pRab** + **0.5 µg UDD1** + **0.5 µg UDD2** + **0.5 µg 8G10** antibody and continue with the second round of immunoprecipitation for another **02:00:00** at **4 °C** on an end-to-end rotator.

63 2m

Centrifuge at **1500 x g, 4°C, 00:02:00** .
Collect the flow through and transfer it to new 1.5 ml Eppendorf tubes.

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

This flow through can also be used for subsequent enrichment of a different analyte or second round of IP or phosphopeptides using TiO2.

64

Add **400 µl 1X IAP buffer** to the beads and carefully transfer it to the beads that are kept **On ice** from 1st round of immunoprecipitation in step 61.

65

Add another **600 µl 1X IAP buffer** to the beads and gently wash tubes by inverting up-and-down for about 20 times.

This can be easily done by keeping samples on an ice-block and cover it with silver foil. Now complete wash by inverting up-and-down steps for all tubes at once. Use this technique for all subsequent wash steps.

66 5m

Centrifuge tubes at **1500 x g, 4°C, 00:03:00** . Place tubes on ice block and allow the beads to settle for at least **00:02:00** before aspirating the supernatant.

67 5m

Repeat washing by adding **1 mL ice cold 1X IAP buffer** and centrifuge tubes at **1500 x g, 4°C, 00:03:00** . Place tubes on ice block and allow the beads to settle for at least **00:02:00** before aspirating the supernatant.

68 5m

Wash the beads with **1 mL ice-cold 1X PBS** , Centrifuge tubes at **1500 x g, 4°C, 00:03:00** . Place tubes on ice block and allow the beads to settle for at least **00:02:00** before aspirating the supernatant. (1/2)

68.1 5m

Wash the beads with **1 mL ice-cold 1X PBS** , Centrifuge tubes at **1500 x g, 4°C, 00:03:00** . Place tubes on ice block and allow the beads to settle for at least **00:02:00** before aspirating the supernatant. (2/2)

69 5m

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Wash the beads with **1 mL ice-cold Milli-Q water** , Centrifuge tubes at **1500 x g, 4°C, 00:03:00** . Place tubes on ice block and allow the beads to settle for at least **00:02:00** before aspirating the supernatant.

70

After the final wash, centrifuge and aspirate all of the Milli-Q water and add **50 µl 0.2% (by vol) TFA** to the beads. Gently tap for proper mixing and incubate on a Thermo mixer at **1250 rpm, Room temperature , 00:10:00** .

71 2m

Centrifuge at **1500 x g, Room temperature , 00:02:00** and collect the eluate into a new collection tube with a gel loader tip.

72 10m

Repeat the elution step by adding another **50 µl 0.2% (by vol) TFA** followed by incubation at **Room temperature** for **00:10:00** and centrifuge and collect the supernatant into the previous collection tube and proceed with C18 stage-tip clean up.

C18 Stage-tip 17m

73 Prepare single layer of C18 stage-tip using 16-gauge syringe needle as shown in figure 2.

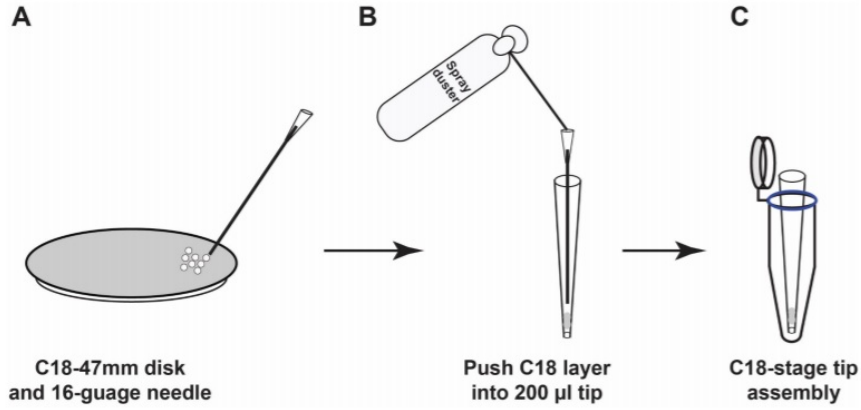


Fig. 2) Homemade C18 stage-tip assembly:
A) Punch a C18 layer using 16 gauge needle
B) Push the C18 layer into a 200 µl pipette tip using a spray duster
C) Stage the tip using PTFE-O ring into a 1.5 ml Eppendorf tube for desalting.

74

Add **80 µl 100% (by vol) Acetonitrile** to the C18 stage-tip.

Note this is required to activate the C18 resin.

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

75 2m

Centrifuge at **2000 x g, Room temperature , 00:02:00** . Discard flow through.

76

Add **80 µl Solvent-A1 0.1% (by vol) TFA (by vol)** .

Note this is required to equilibrate the C18 resin.

77 2m

Centrifuge at **2000 x g, Room temperature , 00:02:00** . Discard flow through. (1/2)

78 2m

Centrifuge at **2000 x g, Room temperature , 00:02:00** . Discard flow through. (2/2)

79 Load the acidified peptide digest from step 72 to Stage-tip.

80 5m

Centrifuge at **1500 x g, Room temperature , 00:05:00** .

81 Reapply the flow through to the C18 stage-tip column and repeat this step. (1/2)

81.1 Reapply the flow through to the C18 stage-tip column. (2/2)

82 2m

Add **80 µl solvent-A1 (0.1% (by vol) TFA v/v)** , and centrifuge at **2000 x g, Room temperature , 00:02:00** . Discard flow through. Repeat again. (1/2)

2m

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

82.1

Add **80 µl solvent-A1 (0.1% (by vol) TFA v/v)** , and centrifuge at **2000 x g, Room temperature , 00:02:00** . Discard flow through. (2/2)

83 Elute peptides absorbed to C18 column by placing the stage-tips into **new** 1.5 ml low binding tubes.

84

Add **30 µl Elution buffer (Solvent B1- 50% (by vol) acetonitrile in 0.1% (by vol) TFA)** .

85 4m

Centrifuge at **1500 x g, 00:02:00** . Add another **30 µl Elution buffer (Solvent B1- 50% (by vol) acetonitrile in 0.1% (by vol) TFA)** and repeat the centrifugation at **1500 x g, 00:02:00** .

86 Immediately snap freeze on dry ice the eluates and vacuum dry the samples using a Speed-Vac.

87

Store samples in **-80 °C** freezer until the LC-MS/MS analysis.

Citation: Raja S. Nirujogi, Dario Alessi (05/06/2021). Sample preparation protocol for targeted mass spectrometry analysis of pRabs, total Rabs, LRRK2-pS910, pS935 and total LRRK2. https://dx.doi.org/10.17504/protocols.io.busynwfw

This is an open access protocol distributed under the terms of the **Creative Commons Attribution License** (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited