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Expression and purification of PPM1H phosphatase

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1 Works for me Share dx.doi.org/10.17504/protocols.io.bu7wnzpe

asap

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ABSTRACT

A subset of small GTPases of the Rab family including Rab8A (Uniprot: P61006) and Rab10 (Uniprot: P61026) have been identified as substrates of the Leucine Rich Repeat Kinase 2 (LRRK2; Uniprot Q5S007)) (Steger et al., 2016). Recent work has revealed that Protein Phosphatase PPM1H (Uniprot Q9ULR3) is a cellular phosphatase that efficiently dephosphorylates LRRK2 phosphorylated Rab proteins (Berndsen et al., 2019). In order to study in detail the kinetics, mechanism and structure of PPM1H, it is necessary to produce milligrams of pure PPM1H protein. Here we describe in detail the method we use to produce milligram quantities PPM1H and several mutants.

ATTACHMENTS

di2hbgrdf.pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

Protein Phosphatase M1H, LRRK2, PPM1H purification

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50134

MATERIALS TEXT

Materials:

Cells:

Competent BL21(DE3)

Plasmids:

Polyclonal Antibody - PPM1H **MRC PPU Reagents and**

- **Services Catalog #DU62835**
- **pET15b 6HIS SUMO PPM1H 33-end (available from mrcppureagents.dundee.ac.uk Order no: DU61331)**

Consumables:

S.O.C.

- Medium **Thermofisher Catalog #15544034**

LB

- broth **Merck Catalog #1.10285.5000**

CARBENICILLIN

- DISODIUM **Formedium Catalog #CAR0025**

IPTG Dioxane Free –

- 25g **Formedium Catalog #IPTG025**

Prepare [M]**1 Molarity (M)** stock solution in H2O.

-

Tris(hydroxymethyl)aminomethane (TRIS Trometamol) 99.8-100.5% AnalaR® NORMAPUR® analytical reagent **VWR Chemicals Catalog #103157P**

-

Glycerine ≥99.5% AnalaR® NORMAPUR® ACS analytical reagent redistilled **VWR Chemicals Catalog #24388.320**

2-

- Mercaptoethanol **Merck Catalog #8057400250**

Magnesium chloride hexahydrate **Sigma**

- **Aldrich Catalog #M2670**

Prepare [M]**1 Molarity (M)** stock solution in H_2O.

Sodium chloride ≥98% TECHNICAL **VWR**

- **Chemicals Catalog #27788.366**

Prepare [M]**5 Molarity (M)** stock solution in H_2O.

Imidazole **Sigma**

- **Aldrich Catalog #56750**

Prepare **50 mL** of a [M]**1 Molarity (M)** imidazole solution (**3.4 g**) and adjust the pH to 7.5 using **1 mL** 37% HCl.

LEUPEPTIN HEMISULPHATE **Apollo**

- **Scientific Catalog #BIMI2183**

Prepare a [M]**10 mg/ml** solution in 50% ethanol and keep at **-20 °C** .

-

4-(2-AMINOETHYL)BENZENESULPHONYL FLUORIDE HYDROCHLORIDE **Apollo Scientific Catalog #BIMB2003**

Prepare a [M]**0.5 Molarity (M)** solution in 50% ethanol and store at **-20 °C** .

Recombinant Protein - SENP1 (415 - 647) **MRC PPU Reagents and**

- **Services Catalog #DU39129**

Poly-Prep® Chromatography Columns Pkg of 50 #7311550 **Bio-rad**

- **Laboratories Catalog #7311550**
- **5 mL** , **10 mL** and **25 mL** polystyrene pipettes (Greiner or Sarstedt)
- **15 mL** and **50 mL** PP centrifuge vials (Greiner, Falcon, Sarstedt)

InstantBlue® Coomassie Protein Stain (ISB1L)

(ab119211) **Abcam Catalog #119211**

- **200 µl** and **1000 µl** pipette tips
- Amicon Ultra-15 3000 Da MWCO centrifuge concentrators.

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SnakeSkin™ Dialysis Tubing, 3.5K MWCO, 22 mm **Thermo Fisher Catalog #68035**

- **2 mL** PP deep well plates for fraction collections with the Äkta Pure. (Greiner)
- Cobalt NTA-Resin (Expedeon, now Abcam, also from Takara or ThermoFisher)

Gel Filtration Standard **BIO-RAD Catalog ##1511901**

Buffered solutions:

Cell collection buffer (make fresh):

A	B
Tris pH 7.5	50 mM
NaCl	150 mM
Imidazole	10 mM
β-mercaptoethanol	7 mM
MgCl2	2 mM
AEBSF	1 mM
Leupeptin	10 µg/ml

Wash buffer:

A	B	C	D
Tris pH 7.5	50 mM		
NaCl	400 mM		
Glycerol	5%		
Imidazole	10 mM		
β-mercaptoethanol	7 mM		
MgCl2	2 mM		

Elution buffer:

A	B
Tris pH 7.5	30 mM
NaCl	250 mM
Glycerol	5%
Imidazole	300 mM
β-mercaptoethanol	5 mM
MgCl2	2 mM

Dialysis buffer:

A	B
Tris pH 7.5	50 mM
NaCl	250 mM
Glycerol	10%
β-mercaptoethanol	7 mM
MgCl2	2 mM

SEC-buffer:

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A	B
Tris pH 7.5	50 mM
NaCl	200 mM
Glycerol	5%
MgCl2	2 mM
β-mercaptoethanol	7 mM
Brij-35	0.015 %

Equipment:

- **20 µl** , **200 µl** and **1000 µl** Gilson pipettes
- Pipette aid / pipettor
- Infors Bacterial Incubator with Platform for **2 L** conical flasks
- 12 x **2 L** conical flasks for growing E.coli BL21 cells
- Beckman J6 centrifuge with **1 L** centrifuge rotor 4.2
- Beckman Avanti Centrifuge with JA 30.50 rotor
- Eppendorf 5810 R centrifuge
- 12 x **1 L** Beckman centrifuge buckets / pots with lids

  50 mL Polypropylene Bottle with Screw-On Cap 29 x 104mm - 25Pk **Beckman**
- 8 x **Coulter Catalog #357003**
- Probe Sonicator (cell disruptor)
- Stuart Roller Mixer SRT9
- Äkta Purifier (GE-Healthcare Life Sciences) or Äkta Pure (Cytiva)
- Superdex 200 column XK16/60 (Cytiva, formerly GE-Healthcare-Life Sciences)
- WPA cell densitometer (Fisher, VWR)

Transformation of plasmid into competent bacteria 8h 30m 50s

1 30m

Mix **10 µl** of pET15b 6HIS-SUMO-PPM1H plasmid (around **50 ng/µl**) with **50 µl** - **100 µl** of the competent BL21(DE3) cells and incubate **On ice** for **00:30:00** .

2 50s

Transfer the vial to a heat block equilibrated at **42 °C** and leave for **00:00:50** .

3

Transfer the vial back into ice and add **1 mL** SOC medium and mix gently.

4 4h

Incubate for **04:00:00** at **37 °C** for recovery.

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5

Plate **0.1 mL** of the transformation onto a LB broth/ agar plate supplemented with **50 µg/ml** Carbenicillin.

6 4h

Leave the plate **Overnight** in a **37 °C** incubator.

Overnight culture 4h

7

Using a **200 µl** sterile pipette tip, remove one of the colonies from the plate and drop the tip into **100 mL** LB broth medium, supplemented with **50 mg/L** Carbenicillin.

8 4h

Incubate at **37 °C** with **180 rpm** - **200 rpm** rotational shaking **Overnight** .

The culture medium should become totally opaque in the morning.

Set up cells and induce expression 12h

9 Decant 3 x **1 L** LB broth medium into 3 x **2 L** conical flasks.

10

Supplement each flask / litre with **1 mL** of **50 mg/ml** Carbenicillin.

11 4h

Mix and add **10 mL** - **25 mL** of the **Overnight** culture into each flask.

12 4h

Incubate for **04:00:00** at **37 °C** , using an Infors Shaker-Incubator set at **200 rpm** .

13 Sample two or three of the expressions by removing **1 mL** medium and comparing the optical density at **600 nm** with fresh LB medium, using a WPA cell densitometer or a spectrometer.

14

When the OD_{600} has reached 0.7-0.9, change the temperature setting of the Infors incubator to **16 °C** and incubate the cells for at least another hour, all the while shaking at **200 rpm** .

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15

When the flasks have cooled down to **20 °C** or lower, induce PPM1H expression by supplementing the medium with **0.025 Milimolar (mM)** IPTG.

This is achieved by adding **25 µl** of a **1 Molarity (M)** IPTG stock solution to each litre.

16 4h

Leave the cells to express the protein for **Overnight** at **16 °C** .

Collection of cells and preparation of lysate 4w 0d 1h 15m

17 The following morning prepare **0.5 L** of cell collection buffer and chill **On ice** .

18 25m

Decant the content of the **2 L** conical flasks into **1 L** Beckman centrifuge pots, close the pots with their screw-cap lids and sediment the cells by centrifugation for **00:25:00** at **4 °C** at **4200 rpm** using a Beckman J6 centrifuge with the 6 x **1 L** rotor (4.2).

19 Collect and open the pots and carefully decant the spent supernatant medium back into the flasks.

The flasks can now be sent for cleaning and autoclaving. The cell sediment in the pots is expected to have a volume of **3 mL** – **5 mL** .

20

Add **18 mL** of cold cell collection buffer to the sediment.

21

Transfer the **1 L** pots to the Infors incubator and set the temperature to **14 °C** and the rotation to **110 rpm** .

22 30m

Leave the pots for **00:30:00** , after which time the cell sediment should have completely resuspended.

23

Pool all suspensions into one of the pots using a **25 mL** pipette and a good pipettor. If any of the sediments has not well resuspended, pipette up and down close to the bottom of the pots.

It is very useful at this stage to supplement the NaCl concentration to **250 Milimolar (mM)** and add glycerol

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to 5% (by vol) final concentration. It is important to add the NaCl and glycerol after the resuspension step and not before as this interferes with the resuspension.

24 20m

Once the NaCl and glycerol has been mixed in, **45 mL** samples are aliquoted into **50 mL** centrifuge vials and freeze them in liquid nitrogen for at least **00:20:00** .

25 Store the vials at **-20 °C** .

The freezing and subsequent thawing step breaks up the cells and improves yield. Vials can be stored at -20ºC for up to 4 weeks.

Preparation of cell lysate and pulldown of His-SUMO-PPM1H on Cobalt-resin 1h 58m 30s

26 Slowly thaw the vials with the cell suspension in cold water.

27 30s

After thawing chill suspension **On ice** and then sonicate, using a probe sonicator (Cell disruptor). Settings: 6 – 8 pulses of **00:00:15** with **00:00:15** pauses. Set the amplitude to 50%.

28 25m

Transfer the sonicated suspension into **50 mL** Beckman centrifuge vials and sediment the insoluble material by centrifugation for **00:25:00** at **40000 x g** and **4 °C** using a JA25.50 or a JA30.50 rotor in a Beckman Avanti centrifuge.

29

Recover the supernatant by carefully decanting it into **50 mL** conical centrifuge tubes. During the centrifugation step equilibrate **3.0 mL** Cobalt-NTA-resin (**6 mL** of a 50% slurry by vol, sufficient for a 3 L expression) by washing it three times with Milli Q water and once with cell collection buffer.

30 1h 30m

Add a **1 mL** of a 50% slurry (by vol) of the washed Cobalt-NTA-resin in collection buffer to each **50 mL** vial with the lysate and incubate the mix on a Roller Mixer for **01:30:00** in a cold room set at **4 °C** .

31 Avoid excessive agitation and especially formation of foam.

32 In the meanwhile, prepare the washbuffer.

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33 2m

Sediment the Cobalt-NTA-resin by centrifugation at **1000 x g** for **00:02:00** at **4 °C** using a Eppendorf R5810.

34

Remove the lid and carefully remove the supernatant containing the depleted lysate using a **10 mL** pipette, being careful not to disturb the resin.

35

Add **3 mL** of wash buffer. Prepare a **1000 µl** pipette tip by removing **5 mm** – **7 mm** from the pointed end using scissors. This allows it to be used to facilely resuspend the agarose.

36 Resuspend the Cobalt-NTA-resin using a P1000 with such a modified blue tip and combine the resin into a **15 mL** centrifuge vial.

37

Wash out any remaining agarose from the large vials with **1 mL** of wash buffer and pool with the first batch to maximise recovery.

38 1m

Fill the **15 mL** vial to the top with wash buffer, mix well and sediment resin by centrifugation at **1000 x g** for **00:01:00** using an Eppendorf 5810 R centrifuge.

39

Remove the wash buffer with a thin vacuum line and replace with fresh wash buffer. Repeat this step 5 times in total to thoroughly wash the resin.

40 Remove all wash buffer without disturbing the agarose bed.

41 Resuspend the resin into **3 mL** wash buffer, transfer into a BIORAD Polyprep column and let the buffer run out.

42

Add **1 mL** of elution buffer and let the void of the bed run out.

43 Then elute the protein with **5 mL** elution buffer into a **15 mL** centrifuge vial.

This should provide **5 mL** of protein solution of **3.0 mg/ml** - **5.0 mg/ml** (**9 mg** - **15 mg** total protein).

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44 Keep the sample volume small to avoid unnecessary protein concentration steps before the subsequent size exclusion.

Removal of the His-SUMO-tag with His-SENP1 (415-644) 1m

45 

For certain applications it may be desirable to remove the N-terminal 6His-SUMO-tag. This can be achieved by incubation of the eluted PPM1H with His-SENP1 (415-644).

For each **10 mg** of eluted PPM1H, mix with **1 mg** of His-SENP1.

46  1m

Transfer the proteins into a Snake Skin dialysis tube and dialyse **Overnight** against **5 L** of dialysis buffer.

Dialysis removes 95% of the imidazole and also concentrates the PPM1H, due to the higher glycerol concentration in the dialysis buffer.

47 Recover the protein the next day and remove the 6-His-SUMO-tag and the His-SENP1 by letting the proteins drip through a **2 mL** bed of Cobalt-NTA-resin, equilibrate in dialysis buffer.

48 

Wash the bed with **2 mL** of dialysis buffer and pool this wash with the flow through to maximise recovery.

Further purification by Size Exclusion Chromatography

49 In order to further improve the purity of the PPM1H and to separate the active dimer from less active tetramer, employ SEC using a SD200 column.

50 Equilibrate a Superdex 200 XK 16/60 format in 3 column volumes of SEC buffer at a flowrate of **1.2 ml/min** using an Äkta Purifier or an Äkta Pure.

51 Apply **1.5 mL** aliquots of the PPM1H sample (typical concentration of **3.0 mg/ml** - **4.5 mg/ml**) to the column at a flowrate of **1.2 ml/min** and collect **1.2 mL** fractions between **44 mL** (void) to **110 mL** .

52 Elute the tetrameric PPM1H as a shoulder between **60 mL** and **75 mL** .

53 The dimeric PPM1H elutes as a peak between **75 mL** and **87 mL** (apex at **81 mL**).

We do not observe a monomer of PPM1H.

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54 Pool and concentrate the fractions from **77 mL** to **87 mL** to obtain the dimeric PPM1H.

A typical elution chromatogram for PPM1H is shown on Figure 1a and for PPM1H (33-end).

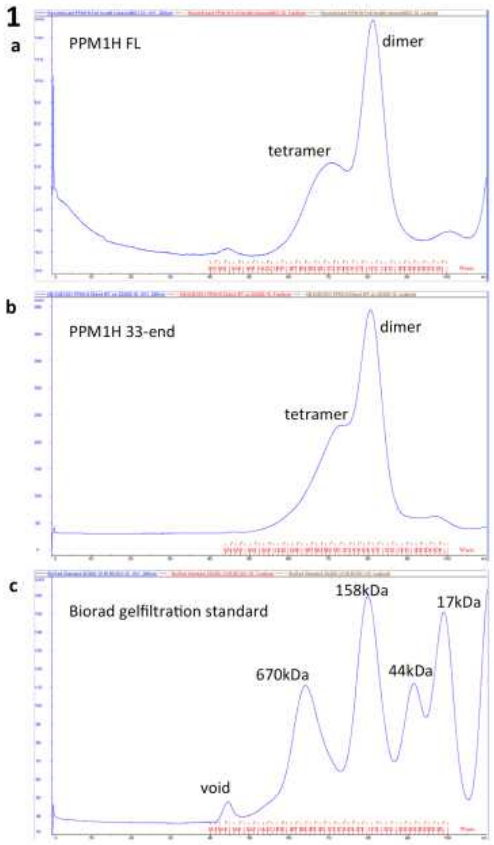

Figure 1 Chromatography profiles of PPM1H, PPM1H (32-end) and the BIORAD gel filtration standard, separated on a Superdex 200 XK 16/60 column, using Unicorn 4.1 software on an Äkta Purifier.

Quality control

55 Separate **3 µg** of the protein on a Bis-Tris 4% - 12% SDS-polyacrylamide gel and stain with Instant Blue Figure 2.

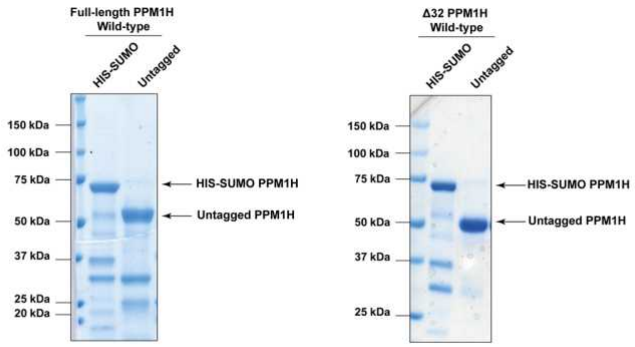

Figure 2 4% - 12% SDS polyacryl amide gel electrophoresis and stained with Instant Blue Coomassie stain. Left panel: His-SUMO-PPM1H and PPM1H. Right panel: His-SUMO-PPM1H (33-end) and PPM1H (33-end) respectively.

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