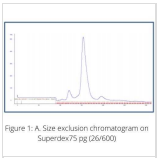

Jul 31, 2021

Expression and purification of Tribolium castaneum orthologue of PINK1

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ABSTRACT

Mutations in PINK1 (protein kinase) and Parkin (ubiquitin E3 ligase) have been linked to familial early-onset Parkinson's disease. PINK1 phosphorylates ubiquitin and the N-terminal ubiquitin-like domain (Ubl) of Parkin at a conserved Serine65 residue in both proteins to initiate mitophagy. Direct measurement of human PINK1 kinase activity has been challenging due to poor expression and low activity in vitro. The discovery of catalytically active insect PINK1 orthologues including Tribolium castaneum PINK1 (TcPINK1) and Pediculus humanus corporis PINK1 (PhcPINK1) has enabled elaboration of robust assays for measurement of PINK1 kinase activity (Woodroof et al., 2011). These assays can be employed for the study of human pathogenic PINK1 mutations and in vitro structural and functional studies of PINK1 (Kumar et al., 2017). Herein, we describe in detail a protocol for expression and purification of TcPINK1 for crystallisation as well as for in vitro characterisation.

ATTACHMENTS

Expression of TcPINK protocol (163 - 334).pdf

DOI

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PROTOCOL CITATION

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MATERIALS TEXT

Competent cells

- BL21 codon plus (DES) RIPL (Stratagene).

Plasmids

- pET 6His sumo codon optimised Tribolium PINK1 150-end ΔI261-L270 S205E E527A K528A
(Available from mrcppureagents.dundee.ac.uk Order no: DU51904).

Consumables

IPTG Dioxane Free –
25g **Formedium Catalog #IPTG025** Prepare 1M stock solution in H_2O

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. (Formedium: 7365-45-9)

N-(2-HYDROXYETHYL)PIPERAZINE-N-3-PROPANESULPHONIC ACID **Apollo Scientific Catalog #BIH0601**

D-( )-Sucrose AnalaR NORMAPUR® analytical reagent **VWR Chemicals Catalog #27480.360**

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

Magnesium chloride hexahydrate **Sigma** – **Aldrich Catalog #M2670** prepare 1M stock solution in H_2O.

TRIS(2-CARBOXYETHYL)PHOSPHINE HYDROCHLORIDE **Apollo Scientific Catalog #BIT0122**

Sodium chloride 99.5-100.5% AnalaR NORMAPUR® ACS Reag. Ph. Eur. analytical reagent **VWR Chemicals Catalog #27810.364**
Prepare 5 M stock solution in H_2O.

Deoxyribonuclease I from bovine pancreas **Sigma** – **Aldrich Catalog #DN25-1G**

Ampicillin (Formedium: 3483-12-3)

Imidazole **Sigma**
Aldrich Catalog #I0125 Prepare **1 Molarity (M)** imidazole solution and adjust the pH to 8.0 using 37% HCl.

LEUPEPTIN **Apollo Scientific Catalog #BIMI2183** Prepare a **10 mg/ml** solution in 50% ethanol and keep at **-20 °C** .

Pefabloc **Sigma**
Aldrich Catalog #76307 Prepare a **0.5 Molarity (M)** solution in 50% ethanol and store at **-20 °C** .

S.O.C
Medium **Thermofisher Catalog #15544034**

SNEP1 Protease (purified in house)

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Poly-Prep® Chromatography Columns **Bio-rad**

5 ml Empty **Laboratories Catalog #7311550**

Empty Disposable Gravity Flow Columns 60 mL with 50 µm frits **Contributed by users Catalog #12-0279-020**

Dialysis Tubing 3.5K MWCO 22 mm **Thermo Scientific Catalog #68035**

InstantBlue® Coomassie Protein Stain (ISB1L) (ab119211) **Abcam Catalog #119211**

Vivaspin 20, 30,000 MWCO PES, 48pc
Centrifuge concentrator
Sarturious VS2022

Nickel sepharose beads (from mrcppureagents.dundee.ac.uk).

Media and reagents

Luria-Bertani (LB) medium (Thermofisher)

A	B
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	800 ml

Make up to 1L mark in a measuring cylinder and mix.

*Autoclave at **121 °C** for **00:20:00** .

Allow to cool and its ready for use.

Terrific Broth Growth Medium (TB)

Terrific Broth Growth Medium (TB) - 1L TB-b solution

A	B
Potassium dihydrogen phosphate (KH2PO4)	23.14 g
di-Potassium hydrogen orthophosphate trihydrate (K2HPO4.3H2O)	125.41 g
deionised sterile or distilled water	1L

Terrific Broth Growth Medium (TB) - 1L TB medium

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A	B
Oxoid tryptone	12 g
Merck yeast extract	24 g
50% Glycerol	8 ml
Deionised deionised or distilled water or Distilled water	900 ml

*Autoclave at **115 °C** for **00:15:00** .

Let it cool down to **50 °C** and add **100 mL** of the previously prepared TB-b.

This can be scaled up as needed.

Lysis/Binding buffer

A	B
EPPS	50 mM
NaCl	500 mM
Glycerol	5%
Sucrose	3%
Imidazole	5 mM
TCEP pH 8.6	0.5 mM

Wash buffer:

A	B
EPPS	50 mM
NaCl	500 mM
Glycerol	5%
Sucrose	3%
Imidazole	20 mM
TCEP pH 8.6	0.5 mM

Elution Buffer:

A	B
EPPS	50 mM
NaCl	500 mM
Glycerol	5%
Sucrose	3%
Imidazole	400 mM
TCEP pH 8.6	0.5 mM

Dialysis buffer:

A	B
HEPES pH 7.5	30 mM
NaCl	500 mM
Glycerol	5%
Sucrose	3%
TCEP	0.5 mM

PEG dialysis buffer:

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A	B
PEG 20,000	20 - 30%
HEPES pH 7.5	50 mM
NaCl	500 mM
Glycerol	5%
Sucrose	3%
TCEP	0.5 mM

SEC-buffer:

A	B
HEPES	25 mM
NaCl	300 mM
Glycerol	5%
Sucrose	3%
TCEP pH 7.5	0.5 mM

Equipment

- Infors Bacterial Incubator with Platform for 2L conical flasks.
- 12 x 2L conical flasks for growing cells.
- Beckman J6-MI centrifuge with 1L centrifuge rotor 4.2
- Beckman Avanti-J25 Centrifuge with JA 25.50 rotor
- Eppendorf 5810 R centrifuge.
- 12 x 1L Beckman centrifuge buckets / pots with lids.
- 8 x 40 ml Beckman Centrifuge tube with lids for JA30.50 centrifuge.
- Sonicator (Branson, Model 102C (CE)).
- Akta prime plus (Cytiva formerly GE-Healthcare-Life Sciences)
- Superdex 75 column (26/600) or any of HR10/30, GL300/10 or XK16/60 (Cytiva, formerly GE-Healthcare-Life Sciences).

Transformation of competent bacteria 2h 30m 50s

1

Mix **1.0 µl** of pET 6His sumo codon optimised TcPINK1 150-end ΔI261-L270 S205E E527A K528A plasmid ([M] **50 ng/µl**) with **50 µl** of the competent BL21(DE3) cells.

2 30m

Incubate **On ice** for **00:30:00** .

3 50s

Heat shock the cells in a water bath at **42 °C** for **00:00:50** .

4

Transfer the vial back onto ice and add **200 µl** SOC and mix gently.

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1h

5 

Incubate for **01:00:00** at **37 °C** for recovery.

6 Plate all of the cells onto a LB-agar plate supplemented with **10 µg/ml** ampicillin.

7 

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

Making overnight culture

8 

Pick a single colony using a sterile pipette tip from the plate and drop the tip into **100 mL** LB medium, supplemented with **10 µg/ml** ampicillin.

9 

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

The culture medium should become totally dense or turbid the following morning.

Protein expression

10 Decant 6 x 1L TB medium into 6 x 2L autoclaved conical flasks.

11 

To each flask add **50 µg** of ampicillin per litre.

Prepare the stock antibiotics as 1000- fold to ensure consistent supplementation.

12 

Then add **10 mL** of the **Overnight** culture into each flask.

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13

Incubate at **37 °C** , using an Infors Shaker-Incubator set at **180 rpm** .

14 4h

After **04:00:00** check the OD_{600} by taking **1 mL** of randomly selected flasks and a fresh TB medium as blank using a WPA cell densitometer or a spectrometer.

15 1h

When the OD_{600} has reached 0.6 - 0.8, lower the temperature of the incubator to **16 °C** and continue the incubation for at least another **01:00:00** .

16 4h

When the temperature drops to **16 °C** , induce protein expression by adding **100 µM** of IPTG (From 1 M stock) and allow the cells to continue to grow for a further 16 - 20 h (usually **Overnight**).

Harvesting of cells 30m

17 The following morning, decant the content of the 2 L conical flasks into 1 L Beckman centrifuge pots.

18 30m

Close the pots tightly and sediment the cells at **4 °C** by centrifugation for **00:30:00** at **4000 rpm** using a Beckman J6 centrifuge with the 6 x 1 L rotor.

19 At the end of the run, carefully decant the supernatant back into the flasks.

The flasks can then be autoclaved and cleaned.

20 The cell pellets (usually between **3 mL** – **5 mL** volume) are then resuspended using the lysis buffer.

21

To do this, **10 mL** of cold lysis buffer is added to the pellet in each of the pot supplemented with **30 µl** of a **10 mg/ml** Leupeptin solution, **60 µl** of a 0.5 M Pefabloc solution and **100 µl** of **1 mg/ml** DNase

22 30m

Transfer the **1 L** pots to the Infors incubator and the temperature still at **16 °C** and the rotation lowered to

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120 rpm . Leave the pots to rotate for 00:30:00 , after which the cell pellet should have completely resuspended.

If there are still some pellet left, pipette up and down close to the bottom of the pots.

23 Transfer the resuspended cells into 50 mL falcon tubes with 30 mL aliquots and flash freeze in liquid nitrogen (this helps to also break up the cells) and stored in -20 °C for short time storage or in -80 °C for longer storage.

Preparation of cell lysate and pulldown 2h 40m

24

Best to prepare all the buffers a day before and keep in the cold room

When you are ready for the purification, slowly thaw the cells suspension on On ice or in cold water.

Make sure the cells are On ice always or best work in the cold room

25 25s Once the cells are resuspended move to lyse the cells using a sonicator or cell disruptor. Sonicate On ice , using a probe sonicator (Settings: 6 – 8 pulses of 00:00:10 with 00:00:15 pauses. Set the amplitude to 45%.

Please note the lysate will warm up slightly, but make sure it does not warm up over 18 °C

26 40m

Transfer the sonicated suspension into 40 mL Beckman centrifuge tubes and collect the cell debris by centrifugation at 4 °C for 00:40:00 at 20.000 x g using a 25.50 rotor in a Beckman Avanti centrifuge.

27

Load 5.0 mL Ni-agarose slurry on a 60 mL gravity column and wash three times with Milli Q water and once with the binding buffer.

5 ml of a 50% slurry, sufficient for a 6 L expression

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28 2h

Add the Ni-agarose slurry to the cell lysate in an appropriate size Duran bottle and incubate the mix on a roller shaker for **02:00:00** in the cold room.

Do not filter the lysate

29

After the incubation period, collect the beads by passing the mixture through a **60 mL** gravity flow chromatography column, wash the beads thoroughly using the washing buffer.

30

Check that the beads are clean by adding **20 µl** of the flow through to **100 µl** of Coomassie.

31 Once clean elute the protein using the elution buffer.

Check that all the protein has been eluted by checking with Coomassie

32 2h

Cleave PINK1 from His-sumo by dialysing the eluted protein against the dialysis buffer using the 3.5 kDa dialysis bag in the presence of His-Snep1 protease **Overnight** in the cold room.

1 mg of His-SENP1 per 25 mg of PINK1

The following day perform negative pull down to get rid of snep1 and sumo.

Negative pull down

33 To remove His-sumo and His-senp1 from the cleaved protein, perform negative pull down.

34

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To do this, still in the cold room, pack a **5 mL** PolyPrep chromatography column with **2 mL** nickel beads, equilibrate with binding buffer and gently by pipetting pass the dialysed protein mixture through the packed Ni-beads.

This traps the Histidine tagged proteins and your cleaved PINK1 protein passes through

35 Collect the flow through into a new falcon tube.

Concentration of the protein 10h

36 10h

This is the tricky bit as the protein tends to aggregate/oligomerises and this make concentrating the protein using the vivaspin concentrator a bit difficult (increased agitation of the protein due to long concentration time).

To concentrate the protein, consider using PEG20,000. To do this, the protein is dialysed against the PEG dialysis buffer. This takes between **05:00:00** to **Overnight** depending on the starting volume

Immerse the dialysis bag with protein in some peg2000 powder will equally do the magic in reducing the volume

The idea is that you want to reduce the volume drastically before moving to vivaspin concentrator to reduce the level of agitation on the protein.

Size Exclusion Chromatography

37

To obtain a crystallization grade protein, size exclusion chromatography is always the last purification step. This can be done on any of the Akta systems at **4 °C** on a superdex75 pg (26/600) or superdex200 pg 16/600 column

Equilibrate the column with 2-column volume of the column size with the SEC buffer.

Before loading the sample clean the Akta sample loop with 2CV of the loop size first with water followed by the SEC buffer.

38 Inject the sample and run the program (usually 1 ml/min flowrate and **3 mL** fractions).

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Using this program on superdex 75 column gives a chromatogram shown in Figure 1a. Fig1A.PNG

Purity check

39 Run 5 µl of fractions within the peak (Figure 1a) on a 12% Tris Glycine SDS-polyacrylamide gel at the end of the run and stain with small amount Instant Blue.

40 After detaining a single band corresponding to ~ 48 kDa should be visible as shown in Figure 1b. Fig1B.PNG

Quantification and storage 3h

41 Pull together the cleanest fractions within the peak and concentrate using the vivaspin 30 kDa cut off to at least 10 mg/ml or less this might take between 02:00:00 - 03:00:00 depending on your initial volume. 5h

Here it is expected that you have less volume, however if your volume is large you might consider using the PEG method to reduce the volume before finishing off with vivaspin concentrator.

42 Make aliquots and flash freeze in liquid nitrogen and stored in -80 °C .

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