Expression and
purification of
recombinant human
Parkin and pSer65
Parkin

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Expression and purification of recombinant human Parkin and pSer65 Parkin

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We use this protocol and it's working

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ABSTRACT

Mutations in PARK2 encoding Parkin are causal for early-onset Parkinson's disease. Parkin is a ubiquitin E3 ligase and is activated by the PINK1 kinase to stimulate ubiquitin-dependent removal of damaged mitochondria via autophagy (mitophagy). The ability to generate recombinant Parkin in its inactive unmodified conformation and activated phosphorylated conformation represent important tools for studying its regulation by PINK1-dependent phosphorylation as well as understanding how disease mutations impact on its function. This protocol describes the expression and purification of full-length human untagged Parkin along with how to make the phosphorylated protein.

In cells the removal of malfunctioning organelles is important for cellular survival. Damaged mitochondria are removed via mitophagy upon activation of the PTEN induced kinase 1 (PINK1) / Parkin pathway. In healthy mitochondria PINK1 is cleaved by PARL and released into the cytoplasm where it is degraded by the N-end rule. In damaged mitochondria depolarisation of the mitochondrial membrane stabilises PINK1, causing it to accumulate on the outer mitochondrial membrane. Stabilised PINK1 phosphorylates Ser65 on both ubiquitin and the ubiquitin like (Ubl) domain of Parkin, this activated Parkin. Active Parkin ubiquitinates outer mitochondrial membrane proteins, leading to ubiquitin chains containing pSer65 ubiquitin. The formation of these chains on the mitochondria identifies it for removal by mitophagy. Loss of activity in either Parkin or PINK1 have been observed in cases of early-onset Parkinson's disease.

Parkin is a RING-in-between-RING (RBR) E3 ubiquitin ligase containing a ubiquitin like (UBL) domain, three RING domains (referred to as RING0, RING1, and RING2 domains), and an in between ring (IBR) domain. Unmodified Parkin exists in an autoinhibited conformation, and phosphorylation of Ser65 on the Ubl domain along with binding to pSer65 ubiquitin causes a significant conformational change that activates Parkin. Previous studies have shown that a small purification tag on the N-terminus of Parkin can lead to constitutive activation, making the production of untagged Parkin an important tool in the study of its regulation and ubiquitylation activity. We describe a protocol for the generation of untagged recombinant Parkin using a His-SUMO tagged fusion construct. In addition we describe the additional steps to produce phospho-Parkin. The yield of Parkin produced varies between 2 and 6 mg, and this is 1/3 lower for pSer65 Parkin. For pSer65 Parkin the stoichiometry of phosphorylation can vary from 60 to 80 %.

MATERIALS

Cells:

- BL21 DE3 pLysS codon plus cells were made chemically competent using the Inoue method.

Plasmids:

- 6His-Sumo-Parkin was inserted into a pCRNT-TOPO pLtetO₂-tetR vector conferring ampicillin resistance. The protocol has also worked on 6His-SumoParkin point mutations inserted into a pET15b vector conferring ampicillin resistance.

Consumables:

- S.O.C. Medium **Thermo Fisher Scientific Catalog #15544034**
- LB Broth **Merck MilliporeSigma (Sigma-Aldrich) Catalog #EM1.10285.5000**
- Carbenicillin - CAR005 Formedium
 - Prepare a 50 mg/ml stock in deionised water just before use
- Isopropyl β-D-1-thiogalactopyranoside (IPTG) - IPTG025 Formedium
 - Prepare a 1M solution in deionised water
- ZnCl₂ anhydrous **Thermo Fisher Scientific Catalog #11497737**
 - Prepare a 0.5M solution in deionised water
- Hydrochloric acid **VWR International Catalog #20252-335**
- 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride **Apollo Scientific Catalog #BIMB2003**
 - Prepare a 0.5M stock in 50 % ethanol in deionised water
- Leupeptin **Apollo Scientific Catalog #BIMI2183**
 - Prepare a 10 mg/ml stock in 50 % ethanol in deionised water
- Sodium hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #795429-500G**
 - Prepare a 5M solution in deionised water
- TRIS base **VWR International Catalog #103157P**
 - Prepare a 1M Tris-HCl pH 7.5 stock in deionised water with the pH adjusted using 37.5 % HCl.
- Sodium chloride **VWR International Catalog #27788.366**
 - Prepare a 5M NaCl stock using deionised water
- Imidazole **Merck MilliporeSigma (Sigma-Aldrich) Catalog #56750**
 - Prepare a 1M stock solution in deionised water, to pH ~7.5

- Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) **Contributed by users Catalog #E6635-500G**
 - Prepare a 0.1M stock at pH 7.5 in deionised water by the addition of 5M NaOH
- Glycerol **VWR International Catalog #24388.320**
- Magnesium chloride hexahydrate **Contributed by users Catalog #M2670-500G**
- Adenosine Tri-Phosphat (ATP) **Abcam Catalog #ab14730**
- Tris(2-carboxyethyl)phosphine hydrochloride **Contributed by users Catalog #BIT0122**
 - Prepare a 0.5M stock solution in deionised water
- Amicon Ultra-15 10'000 kDa MWCO centrifuge concentrators – 10781542 Fisher
- 2ml PP deep well plates for fraction collections with the Äkta Purifier – Greiner
- P200 and P1000 pipette tips
- 50 ml polypropylene tubes – Greiner
- Corning microcentrifuge tubes (polypropylene) **Contributed by users Catalog #CLS3620-500EA**
- His-Senp1

Equipment:

- 20 ul, 200 ul and 1000 ul Greiner pipettes
- Infors Bacterial shaking Incubator with 2L conical flask tray
- 1 x 500 ml conical flask for growing E. Coli
- 12 x 2L conical flasks for growing E. Coli
- 12 x 1 L Beckman centrifuge buckets
- Beckman J6 centrifuge with 1L swinging bucket centrifuge rotor 4.2
- Beckman Avanti centrifuge with JA30.50 rotor
- Benchtop centrifuge – Eppendorf 5810R

- Benchtop cooled microcentrifuge – Thermo Fisher Sorvall Legend Micro 21r Microcentrifuge
- 8 x 40 ml vials with lids for JA30.50 rotor
- Sonicator (cell disruptor)
- Akta purifier/ Pure – Cytiva
- Superdex SD200 16/600 pg column – Cytiva

Buffered Solutions:

- Lysis Buffer: 50 mM Tris pH 7.5, 250 mM NaCl, 15 mM Imidazole, 0.5 mM TCEP and 0.1 mM EDTA
- Kinase Buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 10 mM $MgCl_2$, 0.5 mM ATP and 10 % glycerol
- Elution Buffer: 50 mM Tris pH 7.5, 250 mM NaCl, 400 mM Imidazole, 0.5 mM TCEP and 0.1 mM EDTA
- Cleavage Buffer: 50 mM Tris pH 7.5, 200 mM NaCl, 0.5 mM TCEP, and 5 % glycerol
- Size Exclusion Buffer: 50 mM Tris pH 7.5, 200 mM NaCl, 0.5 mM TCEP, and 10 % glycerol

SAFETY WARNINGS

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

Transformation of plasmid into competent bacteria

1d 9h 32m 50s

1 Mix 10 μ L 6His-SUMO-Parkin plasmid stock ($\sim$ 50 ng/ μ L) with 30m

30 μ L competent BL21 DE3 pLysS Codon Plus E⁻ . Coli and incubate On ice for 00:30:00 .

2 Heat shock cells by incubation in a heat block equilibrated at 42 $^{\circ}$ C for 00:00:50 and 2m 50s

return to ice for 00:02:00 .

3 Add 1 mL SOC media , pre heated to 37 $^{\circ}$ C , to the cells and incubate at 37 $^{\circ}$ C for 1h

01:00:00 .

4 Centrifuge the culture at 1000 x g to pellet cells and remove 830 μ L media , leaving

200 μ L .

5 Suspend cells in the remaining media and spread on LB agar plates supplemented with 1d 8h

[M] 50 μ g/mL carbenicillin ., incubate plates at 37 $^{\circ}$ C Overnight [16:00:00].

Starter Culture

16h

6 Using a P200 pipette tip remove a colony from the LB agar plate and transfer to 250 mL LB +

[M] 50 µg/mL carbenicillin in a 500 ml conical flask.

- 7 Incubate the culture 180 rpm, 37°C, 16:00:00 , (180-200 rpm) , the culture should be opaque after incubation.

Protein Expression

- 8 Inoculate 12 x 1 L LB + [M] 50 µg/mL carbenicillin + [M] 250 micromolar (µM) ZnCl cultures each in 2 L conical flasks with 15 mL starter culture and incubate at 37 °C .

- 9 Set the incubator temperature to 15 °C once cultures reach an OD600 of 0.3 - 0.4, after ~3 hours.

- 10 When the incubator temperature is below 20 °C and the culture's OD600 is 0.8- 0.95 add 25 µL IPTG to induce Parkin expression and incubate cultures Overnight at 15 °C .

11

Note

Incubators reduce temperatures at different rates, modify the OD600 at which the incubator temperature is lowered so cultures are at an OD600 of 0.8-0.95 when the incubator reaches 18 °C .

Making Cell Lysate

4m 30s

- 12 Pellet cells by centrifugation at 5020 x g, 4°C, 00:25:00 .

- 13** Remove the media and invert the centrifuge bottles on a stack of paper towels, leave cell pellets to dry for 00:01:00 - 00:02:00 . 2m
- 14** Suspend cell pellets in 25 μ L of ice-cold lysis buffer containing 10 μ g/mL leupeptin and 1 millimolar (mM) AEBSF per 5 ml of cell pellet [the approximate pellet size from 1 L of cell culture].
- 15** Transfer cell suspensions to 50 ml falcon tubes so that each tube contains 30 mL - 40 mL of solution.
- 16** Sonicate suspensions at 50 % amplitude On ice using 00:00:10 pulses with 00:00:20 breaks in between pulses for a total pulse time of 00:02:00 . 2m 30s

Affinity Chromatography 1h

- 17** Pellet cell debris by centrifugation at 35000 x g, 4°C, 00:30:00 and transfer the supernatant to 8 mL Ni-NTA resin in a 500 ml centrifuge pot.
- 18** Incubate the solution with Ni-NTA resin at 5 °C - 7 °C on rollers for 01:00:00 to allow 6His-Sumo-Parkin to bind. 1h
- 19** Centrifuge Ni-NTA resin at 500 x g, 4°C, 00:05:00 to settle the resin, remove the supernatant, and suspend Ni-NTA resin in 40 mL lysis buffer .
- 20** Transfer the suspension to 14 ml polypropylene tubes so to give 2 ml or less NiNTA resin per tube.

21 Settle Ni-NTA resin by centrifugation at 500 x g, 4°C, 00:01:00 and aspirate the supernatant.

22 Wash Ni-NTA resin by suspending in 6x the resin volume of lysis buffer, centrifuge at 500 rpm, 4°C, 00:01:00 to settle the Ni-NTA resin, and aspirate the supernatant.

23 Wash the resin four more times in lysis buffer then twice in cleavage buffer, keeping twice the resin volume of cleavage buffer in the tube after the final wash.

Parkin S65 Phosphorylation

20h

24 Follow these additional steps if making pS65 Parkin, the protein preparation for pS65 Parkin takes an extra day over the wt Parkin protein preparation.

25 Wash Ni-NTA resin six times by suspending in 6x the resin volume of kinase buffer, centrifuge at 500 x g, 4°C, 00:01:00 to settle the Ni-NTA resin and aspirate the supernatant.

26 Leave twice the Ni-NTA resin volume of kinase buffer after the final wash and add 1 mg GST pediculus humanus PINK1 (phPINK1) to the Ni-NTA resin.

27 Suspend the resin by inversion the leave at Room temperature for 03:00:00 without 3h agitation, inverting occasionally, about every 30 minutes.

28 Centrifuge the Ni-NTA suspension at 500 x g, 4°C, 00:01:00 to settle and aspirate the solvent layer.

29 Incubate Ni-NTA resin against 4 x the resin volume with kinase buffer containing 400 mM Imidazole.

30 Transfer Ni-NTA resin to a gravity flow column and collect the flow through and wash the column in $\frac{3}{4}$ resin volume of cleavage buffer.

31 Determine whether the protein has flowed through the column by comparing the colour change between 200 μ L Bradford reagent mixed with 5 μ L cleavage buffer and 200 μ L Bradford reagent mixed with 5 μ L column flow through at the point the buffer reaches the top of the resin bed.

32 Add another $\frac{3}{4}$ resin volume of cleavage buffer if the flow through still has protein present.

33 Check the protein concentration using a Bradford assay, add a 1:50 mass ratio of GST-PhcPINK1 to the elute and dialyse at Room temperature Overnight [16:00:00] against 2 L kinase buffer.

16h

34 Incubate the kinase reaction with 8 mL Ni-NTA resin at 5 °C - 7 °C for 01:00:00 and centrifuging at 500 x g, 4°C, 00:05:00 and aspirate the supernatant.

1h

Checking the Mass of Protein Bound to Resin

1h

35 Suspend the Ni-NTA resin in the leftover cleavage buffer and transfer 100 μ L Ni-NTA resin suspension to a 1.5 ml microcentrifuge tubes.

36 Settle Ni-NTA resin by centrifugation at 500 x g, 4°C, 00:01:00 and aspirate the supernatant using a gel loading tip to reduce the chance of removing Ni-NTA resin.

37 Incubate Ni-NTA resin in 4x the resin volume of elution buffer for 01:00:00 at 4 °C with 1h agitation.

38 Settle Ni-NTA resin by centrifugation at 1000 rpm, 00:01:00 and determine the supernatant protein concentration using a Bradford assay.

39 Use the concentration to determine the protein mass bound to 50 ul Ni-NTA resin and upscale to calculate the total amount of protein bound to the Ni-NTA resin.

Bradford Assay

5m

40 Dilute a 2 mg/mL BSA stock solution in deionised water to give a standard curve of 2 mg/ml, 1.5 mg/ml, 1.0 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml.

41 Transfer two 1 ml aliquots of Bradford reagent per protein sample tested and BSA standard curve sample, and a single aliquot of 1 ml Bradford reagent to use as a blank to 2 ml cuvettes.

42 Add 10 µL of the relevant samples to each Bradford assay aliquot, add 10 µL either elution buffer for undiluted samples, or deionised water for samples diluted 5x or greater to the cuvette used as a blank.

43 Vortex solutions and incubate at room temperature for 00:05:00 and measure absorptions at 595 nm using spectrometer.

5m

- 44 Plot the BSA standard curve, reading off the samples concentration if it is within the linear range of the curve.
- 45 If the sample is outside the linear range repeat the Bradford assay with the sample's dilution adjusted to place it within the linear range.

6His-SUMO Cleavage

16h

- 46 Add a 1:5 mass ratio of His-Senp1 : bound protein mass to the Ni-NTA resin, invert the resin to put into suspension and leave at 4 °C in a tube rack without agitation.

- 47 After an hour suspend the resin again and leave at 4 °C Overnight [16:00:00] without agitation.

- 48 Transfer Ni-NTA resin to a gravity flow column and collect the flow through and wash the column in $\frac{3}{4}$ resin volume of cleavage buffer.

- 49 Determine whether the protein has flowed through the column by comparing the colour change between 200 μ L Bradford reagent mixed with 5 μ L cleavage buffer and 200 μ L Bradford reagent mixed with 5 μ L column flow through at the point the buffer reaches the top of the resin bed.

- 50 Add another $\frac{3}{4}$ resin volume of cleavage buffer if the flow through still has protein present.

- 51 Once there is no protein in the flow through continue to size exclusion chromatography.

Size exclusion chromatography

- 52** Concentrate the eluted fraction to a volume of less than 1.5 ml by centrifuging at 3000 x g, 10°C, 00:15:00 at a time at 10 °C in a 10 kDa cut-off concentration column, mix the solution between each centrifugation to prevent the protein concentration getting too high at the base of the filter.
- 53** Transfer the concentraed sample to a 1.5 ml centrifuge tube and centrifuge at 17000 x g, 00:15:00 , this should leave a small pellet.
- 54** Transfer the supernatant to a fresh 1.5 ml microcentrifuge tube and load into a 2 ml injection loop.
- 55** Resolve on a superdex 200 16/600 column, the Parkin peak generally should elute at 85-95 ml, pS65 Parkin elutes later than wt Parkin.
- 56** Pool fractions under the peak, taking only those close to the centre if very pure Parkin is required.
- 57** Concentrate the pooled fractions to between 1 and 2 mg/ml using a 10 kDa cut off concentration column using a series of centrifugation pulses at 3000 rpm, 10°C, 00:15:00 , mixing the sample after each pulse.
- 58** Aliquot the final protein sample, flash freeze in liquid nitrogen and store at -80 °C until required.

Quality control

- 59 Test protein purity using resolve 3 µg final fraction using a 4 % 20 % polyacrylamide gel using SDS-PAGE.
- 60 Observe Parkin and any possible contaminants by staining using a Coomassie brilliant blue stain.
- 61 Test Parkin activity by performing a Parkin ubiquitination assay.
- 62 Unphosphorylated wt Parkin should have no ubiquitination activity, however it should become active when treated with TcPINK1.

63

Note

It is possible to express Parkin with some activity, the suitability of this protein depends on the final application.