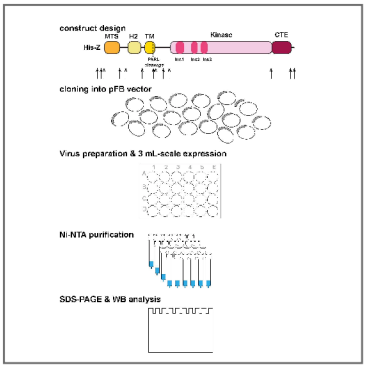

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Protocol status: Working
We use this protocol and it's working

Small Scale purification test for expression of human PINK1 in insect cells

Verena Dederer[1,2,3], Olawale G. Raimi[4], Miratul M K Muqit[4,3], Stefan Knapp[1,2,3], Sebastian Mathea[1,2,3]

[1]Institute of Pharmaceutical Chemistry, Goethe University Frankfurt, Max-von-Laue-Straße 9, Frankfurt 60438, Germany;
[2]Structural Genomics Consortium, Buchman Institute for Molecular Life Science (BMLS), Max-von-Laue-Straße 15, Frankfurt 60438, Germany;
[3]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20815, USA;
[4]Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK

Verena Dederer

ABSTRACT

Purified, recombinant proteins are essential for biochemical characterization and structural studies. However, every protein is unique in its *in vitro* behaviour and thus, establishing a stable expression system and purification protocol can be challenging. Therefore, testing several constructs in parallel may increase the chances of getting a high quality protein prep while minimizing the laborious work. In this protocol we are providing an overview of the main steps in order to clone and test several constructs with varying N- and C-terminal boundaries for expression of human PTEN induced kinase 1(PINK1) in insect cells (this protocol is adapted from 1). PINK1, together with its counterpart Parkin, recognize damaged mitochondria and provoke their degradation by mitophagy, a mitochondrial quality control pathway (for review see 2). Mutations of PINK1 are associated with early-onset Parkinson's disease. Therefore, understanding its structure will help in unravelling its cellular function and hopefully will lead to development of new treatment strategies.

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MATERIALS

Materials

MultiScreen 96well PCR clean-up (Merck Millipore, MSNU03010)
T4 polymerase (NEB #M0203L)
BseRI (NEB # R0581L).

Equipment

DH10bac (Thermo Fisher, 10361012)
TriEx^{TM} Sf9 Cells (Novagen, #71023)
Insect XPRESS, Lonza #BELN12-730Q
Cellfectin^{TM}II (Thermo Fisher #10362100)
3L-Erlenmeyer glass flask
Sonicator + 24-tip horn
Tabletop Centrifuges
Ni-Sepharose beads (Cytiva #17531803).

Lysis buffer

50 mM HEPES pH 7.4, 500 mM NaCl, 0.5 mM TCEP, 5% glycerol
with /without 0.05% digitonin
with/without 300 mM imidazole

Antibodies

anti-PINK1 (#BC100-494, Novus Biologicals)
anti-hexahistidine antibody (#SAB2702220, Sigma-Aldrich).

SAFETY WARNINGS

All experiments are subject of S1 lab regulations.

Section 1: Cloning of human PINK1 constructs into pFB-6HZB

1 Amplify region of interest from human PINK1 gene (OHu25380D; Genscript) with primers endcoding 5'TACTTCCAATCCATG extension for forward and 5'TATCCACCTTTACTG *TCA* extension for reverse primers.

2 DpnI digest template DNA.

3 Purify PCR product using MultiScreen 96well PCR clean-up (Merck Millipore, MSNU03010).

4 Treat purified PCR products with T4 polymerase (NEB #M0203L) in presence of dCTP.

5 Linearize and purify pFB-6HZB using BseRI (NEB# R0581L).

6 Treat linearized vector with T4 polymerase in presence of dGTP to generate complementary sequence overhangs.

7 Anneal vector and insert (ratio 1:4) in 10 µL at Room temperature for 01:00:00. 1h

8 Transform annealing mix into *E. coli.*

9 Plate transformants onto LB agar plates containing 100 µg/mL ampicillin and 5 % sucrose for nick repair and selection.

10 Inoculate liquid culture of successfully growing transformants for plasmid isolation.

11 Validate successful cloning by PCR or sequencing.

Section 2: Baculovirus generation and small-scale test expression

12 Generate bacmids by transforming the plasmids into DH10bac cells.

13 Select for positive transformants by plating cells onto LB agar plates containing 50µg/mL kanamycin, 7µg/mL gentamycin, 10µg/mL tetracycline, 100µg/mL Bluo-gal, and 40µg/mL IPTG.

14 Pick white colonies and inoculate overnight culture for bacmid isolation.

15 Isolate bacmids by alkine lysis and genomic DNA precipitation using sodium acetate precipitation followed by isopropanol wash.

16 Produce recombinant baculovirus by transfecting bacmids into pre-seeded insect cells: 2 mL 0.2 x10^6 cells/mL in 24 well plate.

17 Prepare transfection mix (per well): 1 µL Sample diluted in 49 µL medium and 4 µL Sample diluted in 46 µL Sample

18 Incubate transfection mix for 00:15:00 at Room temperature 15m

19 Add transfection mix to the cells

20 Incubate cells for 168:00:00 at 27 °C for virus production 1w

21 Transfer virus containing supernatant to fresh plate.

22 For protein expression seed 3 mL insect cells (2x10^6 cells/mL) medium in 24 deep-well block.

23 Infect cells with prepared virus containing supernatant (MOI>2).

24 Incubate cells shaking 180 rpm, 27°C, 66:00:00

25 Harvest the cells by centrifugation with 1000 x g, 4°C, 00:20:00 . 20m

26 Freeze cell pellets until further use.

Section 3: Test purification

30m

27 Resuspend cell pellets in 2 mL lysis buffer pH 7.4

28 Lyse cells by sonication (24-tip horn; sonication parameters: 35% amplitude, 5 s pulse / 10 s pause, 3 min total pulse time)

29 Clear lysate by centrifugation 13000 rpm, 4°C, 00:30:00 30m

> **Note**
>
> Keep 10 µL of the supernatant and add 5 µL 4x Laemmli sample buffer for SDS-PAGE analysis

30 Load cleared lysate onto 25 µL pre-equilibrate Ni-Sepharose beads in gravity flow columns

31 Wash beads with 2 mL lysis buffer pH 7.4

32 Elute His6-Z-PINK1 constructs with 50 µL lysis buffer containing [M] 300 Molarity (M) imidazole

Note

Take 10 µL of eluate and add 5 µL 4x SDS sample buffer for SDS-PAGE analysis

33 Perform SDS-PAGE analysis and stain gel with Coomassie stain or further proceed with Western blot analysis.