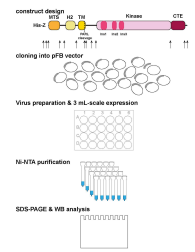




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Small Scale purification test for expression of human PINK1 in insect cells

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We use this protocol and it's working

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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.



Materials

Materials

MultiScreen 96well PCR clean-up (Merck Millipore, MSNU03010)

T4 polymerase (NEB #M0203L)

BseRI (NEB # R0581L).

Equipment

DH10bac (Thermo Fisher, 10361012)

TriEx™ Sf9 Cells (Novagen, #71023)

Insect XPRESS, Lonza #BELN12-730Q

Cellfectin™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 encoding 5'TACTTCCAATCCATG extension for forward and 5'TATCCACCTTTACTGTCA 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 μ L at  Room temperature for  01:00:00 . 1h
- 8 Transform annealing mix into *E. coli*.
- 9 Plate transformants onto LB agar plates containing 100 μ g/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 ×10⁶ 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 (2×10⁶ 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  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 μ L of the supernatant and add  5 μ L 4x Laemmli sample buffer for SDS-PAGE analysis

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

31 Wash beads with  2 mL lysis buffer  7.4

32 Elute His6-Z-PINK1 constructs with  50 μ L lysis buffer containing  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.

Protocol references

1. Preuss F., et al., Enabling pseudokinases as potential drug targets. *Methods Enzymol.* 2022;667:663-683.
2. Agarwal and Muqit MMK , PTEN-induced kinase 1 (PINK1) and Parkin: Unlocking a mitochondrial quality control pathway linked to Parkinson's disease. *Curr Opin Neurobiol* 2022 Feb; 72:111-119.