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We use this protocol and it's working

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Reconstitution of human PINK1 and outer mitochondria TOM complex in yeast

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ABSTRACT

PTEN induced kinase 1(PINK1) is a mitochondria kinase that phosphorylates ubiquitin and Ubl domain of parkin coincidentally at structurally obscured S65 in both proteins and initiate mitophagy. Dysregulation of this process has been implicated in cancer, obesity, cardiac disease, and neurodegenerative diseases (Youle and Narendra, 2011). PINK1 and TOM complex are key regulators in the mitochondrial quality control pathway. Physiologically under ideal condition PINK1 via its MTS forms a complex with the tom complex comprising of seven individual toms (TOMs 5, 6, 7, 20, 22, 40 and 70) going through the pores of TOM40 into the inner mitochondria (IMM) (Rasool et al., 2022). In the IMM PINK1 is cleaved within the MTS region precisely between residues 103 and 104 by two IMM proteases, MPP and PARL. The processed PINK1 is then released back into the cytosol where it is channelled towards proteasomal degradation (Rasool et al., 2022). In a pathological condition however, that's upon mitochondrial damage, PINK1 import is blocked, preventing its processing. This results in PINK1 accumulation at the outer mitochondria membrane (OMM) and the activation of PINK1 through autophosphorylation, which subsequently initiates the mitophagy pathway to prevent the accumulation of reactive oxygen species from damaged mitochondria which could lead to cell apoptosis (Rasool et al., 2022). Here we describe in detail the protocol for making yeast cells stably expressing PINK1 and Human TOMs which has allowed us to characterise PINK1 when co-express with the TOMs.

ATTACHMENTS

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MATERIALS

Yeast cells

The Saccharomyces cerevisiae strain YCE1164 (MATa ade2-1 ura3-1 his3- 11,15 trp1-1 leu2-3,112 can1- 100 bar1Δ::hphNT pep4Δ::ADE2) was generously gifted by Prof. Karim Labib, MRC-PPU unit University of Dundee.

Plasmids

	A	B
	pOR1	Harbours PINK1-3flag + TOM40
	pOR2	Harbours TOM7 + TOM22
	pOR3	Harbours TOM20 + TOM70
	pOR4	Harbours TOM5 + TOM6

Note

All plasmids are available from the mrcppureagents.dundee.ac.uk Order numbers, DU65736, DU65735, DU70212 and DU70130 respectively.

Consumables

- Trichloroacetic acid (TCA; Sigma)
- Glass Beads - 0.5mm dia **BioSpec Products Catalog #11079105**
- Glycerine ≥99.5% AnalaR® NORMAPUR® ACS analytical reagent redistilled **VWR International Catalog #24388.320**
- TRIS(2-CARBOXYETHYL)PHOSPHINE HYDROCHLORIDE **Apollo Scientific Catalog #BIT0122**
- Sodium chloride 99.5-100.5% AnalaR NORMAPUR® ACS Reag. Ph. Eur. analytical reagent **VWR International Catalog #27810.364**

  Prepare [M] 5 Molarity (M) stock solution in H_2O.
- Galactose (Formedium) (Prepare 20% and filtered through ↠← 0.2 µm sterile filter).
- Raffinose (Formedium) (Prepare 20% and filtered through ↠← 0.2 µm sterile filter).
- Tris (hydroxymethyl) aminomethane
- Tris-buffered saline with 0.1% Tween20 detergent (TBST)
- PmlI (NEB)

- AflII(NEB)
- Nhe1(NEB)
- NdeI(NEB)
- Single-stranded DNA (ssDNA) (Prepare 10% stock solution and preheat at least for 00:05:00 before use)

Media and reagents

- Yeast peptone (YP) medium
- Luria-Bertani (LB) medium
- Synthetic complete (SC) agar plates
- Luria-Bertani (LB) agar plates
- Yeast peptone (YPD) agar Plates
- Lysis buffer:

	A	B
	Tris	50 mM
	NaCl	200 mM
	Glycerol	5%
	TCEP pH 8.0 (containing protease inhibitor cocktail)	0.5 mM

Equipment

- Infors Incubator for yeast 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.
- 30ºC incubator
- LiCor (Odyssey CLx)
- Plate reader

Miniprep

1 Before cloning, codon optimise the human genes for yeast expression. To scale up the plasmids, carry out miniprep using the Qiagen miniprep kit following the manufacturer's instructions.

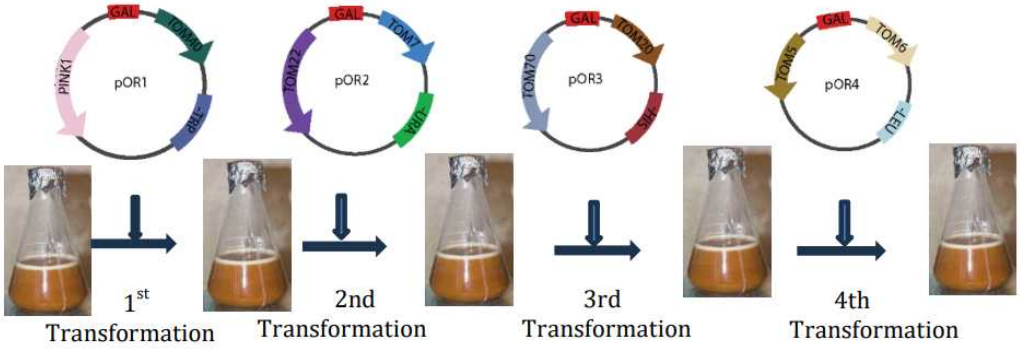

Figure 1: Schematic of sequential transformation of yeast cells. Yeast cells are transformed sequentially with plasmids carrying two genes each of the desired proteins. Each with an appropriate selection marker. Each pair of genes is cloned close to the GAL1_10 promoter. After each transformation, select positive clones on an appropriate synthetic complete medium nutrient drop-out plate.

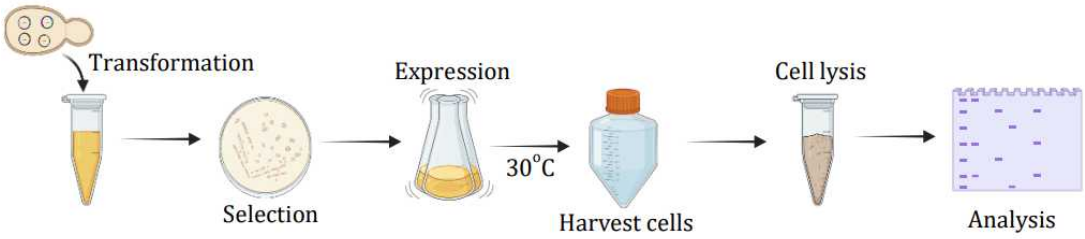

Figure 2: Flow chart of steps in sample preparation.

Transformation of yeast cells

2

Note

Please note that the transformation of cells with the four plasmids should be performed individually until you have a positive clone harbouring all four plasmids (Figure 1 and 2).

To start with the first plasmid, make Overnight culture of MATa cells in 5 mL of YP medium supplemented with 2% glucose. Grow at 30 °C with shaking at 180 rpm.

3 The following morning prepare a 50 mL YP medium containing 2% glucose and inoculate with 5 % overnight culture. Grow at 30 °C with shaking at 180 rpm.

4 While waiting for the right cell density, linearise DNA using the appropriate restriction enzyme for 02:00:00 at 30 °C with shaking using a thermomixer. 2h

5 To do this you need 4 µL of the enzyme buffer in a sterile Eppendorf tube, followed by 2 µL of the restriction enzyme (for pOR1 its Pml1, pOR3 its Nhe1, pOR2 its Ndel1 and pOR4 its Afl11), then 2 µg of DNA and make up to 40 µL with sterile water and allowed to digest for 02:00:00. 2h

Note

After 2 hours the linearised DNA is left On ice waiting for the cells.

6 At an optical density OD_{600} of ~1.6 or a cell count of 2 x 10^7 cells/ml collect cells by centrifugation using tabletop Eppendorf centrifuge (model 5810R) at 4000 rpm for 00:10:00. 10m

7 Decant supernatant and wash cells with sterile water twice each time collect cells by centrifugation.

8 After the last wash resuspend cells with 900 mL of sterile water and from this take 200 µL into a sterile Eppendorf tube and collect cells by centrifugation using a small tabletop centrifuge at highest speed for 00:05:00 . 5m

9 Remove supernatant and keep On ice .

10 To transform the cells with the DNA, in a sterile environment using flame, add 240 µL of sterile Polyetheleneglycol, 36 µL of sterile lithium acetate followed by 50 µL of 10% charged SS DNA and lastly the linearised DNA.

11 Vortex and incubate at 42 °C for 00:20:00 . 20m

12 After the heat shock, collect cells by centrifugation and resuspend with 200 µL of sterile water and plate on a synthetic complete (SC) agar plate with an appropriate dropout supplement. For pOR1 its -TRP, pOR2 its -URA3, pOR3 its -HIS and pOR4 its -LEU.

13 Cells can be split into 30-70% on two plates and a third negative control plate with the untransformed cells.

14 Incubate plates at 30 °C for 48:00:00 . 2d

Screening for positive clone

15 After 48:00:00 colonies should have appeared with the 70% plate containing more dense colonies compared with less dense and well disperse colonies on the 30% plates. 2d

16 Select four colonies and do a test expression for the desired protein.

17 Streak the four colonies separately on a yeast peptone (YP) agar plate divided into four equal partitions and incubate at 30 °C Overnight . 2d

Making overnight culture

18 Make overnight cultures of the four colonies differently by inoculating 5 mL YP medium supplemented with 2% raffinose with a tiny scoop of each clone and grow at 30 °C with shaking at 180 rpm in an Infors shaker designated for yeast.

Protein expression

19 Make a 10 mL culture using YP medium supplemented with 2% raffinose and inoculate with 5% of the overnight culture and grow at 30 °C with shaking as for the overnight culture.

20 At the right optical density (OD_{600} of ~1.7) or cells count of 3 x 10^7 cells/ml, induce protein expression by adding to a final concentration 2% sterile galactose under a sterile condition (close to a flame).

21 Continue growing for a further 10-12 h usually Overnight . 2d

Harvesting of cells

22 Harvest cell by centrifugation at 4000 rpm for 00:10:00 using a tabletop Eppendorf centrifuge. 10m

23 Decant supernatant and place cells On ice .

Protein extraction

24 Perform protein extraction using TCA protein extraction and lyse cells by vortexing in the presence of small amount of glass beads in an Eppendorf tube.

25 First resuspend cells in 200 µL of 20% TCA and transfer into 1.5 ml Eppendorf tube.

26 Add small amount of glass beads and vortex for 00:00:35 . 35s

27 Transfer 50 µL of the lysed cells into a clean and well-labelled tube.

28 Add another 200 µL of 5% TCA, vortex again for 00:00:35 . 35s

29 Take 150 µL and add to the initial 50 µL .

30 Collect precipitated protein by centrifugation at high-speed using refrigerated tabletop centrifuge.

31 Decant supernatant and resuspend protein in a 200 µL lysis buffer.

Protein quantification

32 Quantify protein using BCA. To do this make 1:10 dilution of the cell lysate.

33 Mix 10 µL of these samples along with protein standard (Albumin) with 200 µL of BCA reagent.

34 Incubate at 37 °C for 00:25:00 . 25m

35 Read absorbance at 562 nm and extrapolate concentration using the protein standard curve.

Western blot

3h 50m

36 Perform western blot to check for the expression of the desired proteins, PINK1 activity by blotting for phospho-ubiquitin or other housekeeping genes. To do this, run 20 µg of protein on SDS-PAGE and transfer protein on a nitrocellulose membrane.

37 After the run, block in 5% milk in TBST for 01:00:00 . 1h

38 Wash membrane in TBST (3 x 10 minutes).

38.1 Wash membrane in TBST (3 x 00:10:00) (1/3). 10m

38.2 Wash membrane in TBST (3 x 00:10:00) (2/3). 10m

38.3 Wash membrane in TBST (3 x 00:10:00) (3/3). 10m

39 Incubate in primary antibody Overnight at 4 °C . 10m

40 Next day wash membrane as before with TBST and incubate membrane in secondary antibody at 1h
Room temperature for 01:00:00 .

41 Wash membrane again in TBST and scan membrane on the LiCor machine.

Note

Once a positive clone expressing the proteins of the inserted plasmid has been identified, make glycerol stock of these cells in 20% sterile glycerol and store at -80 °C .

42 Transform these cells with the second plasmid as described above from Transformation of yeast cells section to the Western blot section.

43 Repeat these steps until you have obtained cells with all the four plasmids and capable of expressing all the eight proteins.

44 Also make a glycerol stock of a positive clone expressing all the eight proteins.