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We use this protocol and it's working

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Blue Native PAGE of PINK1-TOM complex in mammalian cells

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ABSTRACT

Autosomal recessive mutations in PTEN-induced kinase 1 (PINK1) are associated with early-onset Parkinson's disease (PD) [1]. Upon inducing mitochondrial depolarization using uncouplers, the full-length human PINK1 stabilizes at mitochondria with the TOM (translocase of the outer mitochondrial membrane) complex, undergoing autophosphorylation and activation [2]. PINK1 plays a crucial role in phosphorylating ubiquitin and the E3 ubiquitin ligase, Parkin, initiating mitophagy for selectively removing damaged mitochondria [3]. To investigate PINK1 mutation impacts on PINK1-TOM complex formation, Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) was done on stable cell lines in PINK1 knockout (KO) HeLa cells expressing doxycycline-induced PINK1/mutants. BN-PAGE substitutes SDS with Coomassie G250, preserving proteins in their native conformation, and utilizes non-ionic detergents for solubilization, ensuring complex integrity during analysis [4, 5]. Studying the PINK1-TOM complex has proved vital for our understanding of PINK1 stabilization on mitochondria. Here, we share our method for resolving protein complexes by blue native PAGE for PINK1-TOM complexes.

ATTACHMENTS

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MATERIALS

Cells

- Doxycycline induced WT-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Plasmid order no. DU43407)
- Doxycycline induced KI-PINK1-3FLAG in PINK1 KO Hela Flip-in cells ((Plasmid order no. DU46669)
- Doxycycline induced empty-3FLAG in PINK1 KO Hela Flip-in cells ((Plasmid order no. DU45919)
- Doxycycline induced L532A L539A L540A-PINK1-3FLAG in PINK1 KO Hela Flipin cells ((Plasmid order no. DU77629)
- Doxycycline induced R83E R88E R98E-PINK1-3FLAG in PINK1 KO Hela Flip-in cells ((Plasmid order no. DU77573)

Consumables

- Phosphate buffered saline (Invitrogen)
- 25G 1” (25mm) syringe needle (Orange)
- BD Plastipak Syringes 1ml x 120
- Benzonase® Nuclease, Merck, Purity >90%
- NativePAGETM 3 to 12% gels (Invitrogen/ThermoFisher)
- NativeMARKTM Unstained protein standard (Invitrogen)
- NativePAGETM 4x Sample Buffer (Invitrogen)
- NativePAGETM 20x Running buffer (Invitrogen)
- NativePAGETM 20x Cathode Buffer additive (Invitrogen)
- NativePAGETM 5% G-250 Sample additive
- 5% Digitonin (Invitrogen)
- 20x NuPAGE transfer buffer (Invitrogen)
- Pierce BCA protein assay kit (ThermoScientific)
- Clarity Western ECL subustrate (biorad)

Antibodies

- PINK1 (D8G3) Rabbit mAb **Cell Signaling Technology Catalog #6946**
- Recombinant Anti-TOMM40 antibody [EPR6932(2)] **Abcam Catalog #ab185543**
- TOMM70 Antibody **Aviva Systems Biology Catalog #OAAN01138**
- Recombinant Anti-TOMM20 antibody [EPR15581-54] **Abcam Catalog #ab186735**

Buffer and reagents

Mitochondrial fractionation buffer: Frozen stock (final Concentration)

	A	B
	HEPES pH 7.5	20 mM
	EDTA	3 mM
	Sodium β-glycerophosphate	5 mM
	Sodium fluoride	50 mM
	Sodium pyrophosphate	5 mM
	Sucrose	250 mM
	Added fresh before use:	
	1x protease inhibitor cocktail tablet (Roche)	
	Sodium orthovanadate	1mM

BN-PAGE buffer

	A	B
	NativePAGE 4x sample buffer	Make 1x buffer
	1% digitonin	As required
	milliQ water	As required
	Benzonase (1-2 U/ul along with 2mM MgCl2)	If smears are observed

Dark Buffer

	A	B
	1x BN-PAGE buffer (1x)	200 ml
	20x Cathode buffer additive	10 ml

Light Buffer

	A	B
	1x BN-PAGE buffer (0.1x)	200 ml
	20x Cathode buffer additive	1 ml

Equipment

- 150mm petri dishes for culturing cells
- VWR Micro Star 21R microcentrifuge for speed up to 17,000 g
- Eppendorf 5430R microcentrifuge for speed up to 20,000 g
- XCell SureLock Mini-Cell gel apparatus (Invitrogen)

Formation of mitochondrial enriched pellet

1 For making mitochondrial enriched fraction first harvest the cells after giving desired treatments (doxycycline for PINK1 induction and Antimycin/Oligomycin for mitochondrial damage).

2 For collection keep plates with cells On ice covered with aluminium foil to provide even cool surface.

3 Wash the cells with PBS and collect the cells with cell scraper.

4 Collect the cells by centrifugation at 800 x g for 00:05:00 at 4 °C . 5m

5 Pellet down the cells at 800 x g for 00:05:00 at 4 °C . For 150 mm plate cell pellet, add 300 µL of mitochondria fractionation buffer. 5m

6 Disrupt cell membranes using a 25-gauge needle by passing through it for 25 times On ice .

7 Clarify lysates by centrifugation at 800 x g for 00:10:00 at 4 °C . 10m

8 Discard the cytoplasmic membrane/nucleus/debris pellet.

9 Isolate supernatant and centrifuge at 17000 x g for 00:20:00 at 4 °C to collect mitochondrial enriched fraction. 20m

10 Try to remove as much buffer as possible from the pellets as it may interfere with the BN-PAGE.

11 Snap-freeze the mitochondrial enriched pellet for Blue native PAGE.

Mitochondrial enriched pellet Solubilization

12 Start with mitochondrial enriched pellets, snap-frozen and stored at -80 °C . A single freeze-thaw cycle has negligible effects on TOMs/PINK1. Whole cells can also be used but the signal is clearer in mitochondrial enriched fraction.

Safety information

Do not freeze-thaw more than once. The lower PINK1 band near 480 kDa is more susceptible to freeze-thaw cycles.

13 Prepare Native-PAGE sample buffer per sample as described in buffer section.

14 Thaw mitochondrial pellets On ice and very gently resuspend them in BNPAGE buffer On ice with 1% digitonin.

Safety information

If you suspect traces of buffer in mitochondrial pellet, first make 1X buffer without digitonin and gently resuspend the pellets. Centrifuge samples at 17000 x g for 00:20:00 and proceed normally.

15 Store On ice and incubate for 00:30:00, gently pipetting up and down 10-15 times every 00:10:00. Digitonin is chosen after optimizing with different reagents. 40m

16 Post-solubilization, centrifuge samples at 20000 x g for 00:30:00 at 4 °C. Transfer the supernatant to a cold Eppendorf, noting a small red pellet of insoluble material. If desired, solubilize this material in 1X SDS-PAGE buffer for further analysis. 30m

17 Quantify the lysate using BCA Protein Assay Kits.

Note

Do not use Bradford protein assay reagent.

18 Make samples of concentration around 1-2 ug/ul, typically loading 15-20 µg of solubilized mitochondrial pellets for one lane of a BN-PAGE gel. For diluting the lysates, make up the samples using 1x BN-PAGE lysis buffer with 1% digitonin. It is important to maintain the detergent percentage constant for proper Coomassie G250 and digitonin raito.

19 Prepare gels in 1X Native PAGE running buffer (20x stock, make up 1 L in Millipore water). Also, prepare light and dark inner chamber buffers. Keep all buffers at 4 °C .

20 Add Coomassie G250 additive to samples (at 1/4 the detergent concentration, i.e., 0.25% if using 1% digitonin). Pipette thoroughly to distribute Coomassie in the sample; the sample will turn a deep blue.

Safety information

Do not heat the samples. Keep the samples On ice the whole time.

21 Set up the gel apparatus in a cold room. Pour a small amount of Dark inner buffer into the inner chamber to check for leaks.

22 Remove any buffer from wells using an aspirator.

Note

Optional: You can give a wash to wells by filling them with clear running buffer and aspirate them.

23 Dry load samples onto the gel and use Native-Page ladder.

24 After loading, fill the inner chamber with 200 mL Dark inner buffer and 1x running buffer in the outer chamber.

25 Run the gel at 7mA constant current and allow the sample to enter the gel until it runs 1/3rd of the total gel. Subsequently, empty both inner and outer buffers, replacing the inner buffer with LIGHT buffer and filling the outer chamber with 1x running buffer again.

26 Continue running the gel at 7mA for 2-3 hours until the full gel is stained.

Transfer

27 Prepare 1x NuPAGE transfer buffer (20x stock) in a quantity sufficient for one transfer tank. Set up the transfer tank as usual.

28 For BN-PAGE, opt for PVDF membranes, as nitrocellulose binds Coomassie irreversibly. Activate the PVDF membrane in 100% methanol for 00:00:30 before placing it over the gel in the transfer tank. 30s

29 Upon completion, place the transfer tank in an ice bucket filled with ice. Run Overnight at ~60mA, limiting voltage to ~15 volts. 30s

Staining and Probing 3h 35m

30 Disassemble the transfer tank. If successful, most or all of the Coomassie staining on the gel will transfer to the PVDF membrane.

31 Destain Coomassie with 100% methanol 2-3 times for 00:05:00 each. A slight Coomassie staining may persist (from the added samples; background staining will be removed). 5m

32 Restain the gel with Ponceau to visualize protein ladders. Mark ladders and scan the blot.

33 Wash Ponceau with TBST (0.1% tween) until fully destained (3 times for 00:10:00 each). It might take longer to destain completely. If needed, incubate in 5% milk in TBST to destain. 10m

34 Block in fresh 5% milk for 01:00:00 (do not use BSA for blocking) and probe Overnight as for a standard Western blot. 2h

35 Wash membranes three times in TBST for 00:10:00 each to remove residual primary antibody. The primary antibody may give different signals depending on how the antibody binding site is exposed in a complex. For visualization of PINK1 in PINK1-TOM complex, CST-PINK1 antibody works best. 10m

36 Utilize HRP-conjugated secondary antibodies. Prepare HRP-conjugated AB (1:5000 in milk), incubate at Room temperature covered for 01:00:00. 1h

37 Remove the secondary antibody and wash three times in TBST for 00:10:00 each and once in TBS. 10m

38 Use ECL to visualize HRP-conjugated antibodies. Prepare the ECL reagent mix (1:1) just before scanning. Pour off TBS on one membrane and add 1 mL ECL mix; immediately scan, adjusting scanning times based on the primary antibody and protein abundance.