Mammalian cell culture and transfection for stable cell lines generation

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

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DISCLAIMER

ABSTRACT

Autosomal recessive mutations in PTEN-induced kinase 1 (PINK1) are linked to early-onset Parkinson's disease (PD) [1]. Upon mitochondrial depolarization, PINK1 activates through autophosphorylation and stabilization on mitochonria [2]. Pink1 phosphorylates ubiquitin and Parkin, triggering mitophagy to remove damaged mitochondria in PD [3]. To delve deeper into the impact of PINK1 mutations, a PINK1 knockout (KO) HeLa cell line was utilized as a model system. Additionally, stable cell lines with mutated PINK1 were established to explore differences in functional activity and the formation of the PINK1-TOM complex between wild-type PINK1 and its mutant variants.

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ATTACHMENTS

Protocols Mammalian cell culture and stable cell line.docx

PROTOCOL REFERENCES

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MATERIALS

1. HeLa Flp-In T-Rex cells and plasmids:

- PINK1 KO HeLa Flip-In T-Rex cells
- Doxycycline induced WT-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU43407)
- Doxycycline induced KI-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU46669)
- Doxycycline induced empty-3FLAG in PINK1 KO HeLa Flip-In cells (DU45919)
- Doxycycline induced L532A-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU60932)
- Doxycycline induced L539A-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU60929)
- Doxycycline induced L540A-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU60930)
- Doxycycline induced L532A L539A L540A-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU77629)
- Doxycycline induced R83A-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU76079)
- Doxycycline induced R88A-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU76082)
- Doxycycline induced R98A-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU76078)
- Doxycycline induced R83E R88E R98E-PINK1-3FLAG in PINK1 KO HeLa Flip-In cells (DU77573)

2. Consumables

1. DMEM high glucose no glutamine **Thermo Fisher Scientific Catalog #**11960044
2. 6 mL in 500 ml media
   L-Glutamine (200 mM) **Gibco - Thermo Fischer Catalog #**25030081
3. Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #**15140122
   (GIBCO); 6 mL in 500 ml media
4. Phosphate buffered saline (Invitrogen)
   10x PBS **Thermo Fisher Scientific Catalog #**AM9624
5. Hygromycin B Gold **Invitrogen** , 0.5 ml in 500 ml media
6. Blasticidin 7.5 mg/ml **InvivoGen** , 1 ml in 500 ml media
7. Zeocin 100 mg/m **InvivoGen** , 1 ml in 500 ml media
8. Fetal Bovine Serum (FBS) (Sigma) **Merck MilliporeSigma (Sigma-Aldrich)** , 10% in media
9. Opti-MEM™ I Reduced Serum Medium **Gibco - Thermo Fischer Catalog #**31985062
10. Doxycycline 1 mg/ml (Sigma-Aldrich), 0.02 ug/ml

11.

Lipofectamine™ 3000 Transfection Reagent **Thermo Fisher Catalog #**L3000001

12. 25G 1” (25mm) syringe needle (Orange)

3. Buffer and reagents:

Mitochondrial fractionation buffer:

	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

Frozen stock (final conc):

	A	B
	Sodium orthovanadate	1 mM
	protease inhibitor cocktail tablet (Roche)	1X

Added fresh before use (final conc):

Lysis buffer:

	A	B
	Tris-HCl (pH 7.5)	25 mM
	EDTA	1 mM
	EGTA	1 mM
	sucrose	0.27 M
	NaF	50 mM
	sodiumpyrophosphate	5 mM
	sodium orthovanadate	1 mM
	sodium β-glycero-phosphate	10 mM
	benzamidine	1 mM
	2-mercapto-ethanol	0.10%

	A	B
	one mini CompleteTMprotease inhibitor cocktail tablet	per 10 ml of lysis buffer
	Triton X-100	1% v/v

Equipment:

- Binder CO_2 Mammalian Incubator
- 150mm Petri dishes for culturing cells
- VWR Micro Star 21R microcentrifuge
- Esco Class II biological safety cabinet
- Grant water bath

Cell Culture

1 Maintain cells at 37 °C in a 5% CO_2 water-saturated incubator.

2 Grow HeLa cells in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (complete media).

3 The cell culture passages usually used are from P10 to P20. The passages are never used above P25.

Maintenance of HeLa Flp-In T-Rex Stable Cell Lines:

4 For HeLa Flp-In T-Rex stable cell lines, use complete media supplemented with blasticidin and zeocin before recombination/transfection for stable cell line generation.

5 Supplement with blasticidin and hygromycin B following recombination/transfection.

Generation of Stable Cell Lines:

4w 5d 0h 15m

6 Achieve doxycycline-induced, stable expression of exogenous protein using the Flp-In T-Rex system according to Invitrogen's instructions, utilizing CRISPR knock-out PINK1 KO HeLa Flp-In T-Rex cells [4]. The exact steps are detailed below.

7 Maintain HeLa PINK1 knock-out Flp-In T-Rex cells in blasticidin and zeocin.

8 Wash cells with PBS wash and switch to complete media 24:00:00 before transfection. 1d

9 Carry out transfection by co-transfecting 0.5 µg integratable hygromycin-resistant pcDNA FRT/TO vector of desired PINK1/mutant with 4.5 µg pOG44 expressing the Flp recombinase using Lipofectamine3000 in 100mm Petri dish [5, 6].

A	B	C	D
A	B	C	D
Tube 1			
	POG44 plasmid	4.5 µg	
	Desired DNA plasmid	0.5 µg	Total DNA = 5µg
	Lipofectamine P3000 reagent	10 µl	
	Opti-MEM	0.5 ml	
Tube 2			
	Lipofectamine reagent	7.5 µl	

	A	B	C	D
		Opti-MEM	0.5 µl	

10 Mix the 2 tubes and keep at RT for 00:15:00 . 15m

11 Add the transfection mix drop by drop in the plate containing HeLa PINK1 knock-out Flp-In T-Rex cells. Keep a plate of untransfected cells as a negative control.

12 After 48:00:00 of transfection, split the cells with around 25% confluency. 2d

13 Once the cells are attached, add fresh complete media supplemented with blasticidin and hygromycin.

14 Maintain the cells with regular media changes every 2-3 days. Remove dying/dead cells when required. If successful, you will see separate colonies growing. Colonies amount varies from 10-50 per plate.

15 Trypsinize surviving colonies after 3-4 weeks of selection.

16 Expand the selected colonies, and induce protein expression with 0.02 µM doxycycline.

Treatment with Mitochondrial Uncoupler:

6h

17 Prepare a 50 mM stock of Antimycin and 6.3 mM of Oligomycin in DMSO, and store at -20 °C .

18 Uncouple mitochondria by treating with 10 µM of Antimycin A and 1 µM of of Oligomycin for 03:00:00 - 06:00:00 , using an equivalent volume of DMSO for control conditions. 9h

Cell Lysis and Mitochondrial Enrichment:

55m

19 **Whole cell lysis**

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

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

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

19.4 Add around 300 µL of Lysis buffer for 100 mm cell plate lysate. Resuspend the cells with lysis buffer containing 1% triton and keep them On ice for 00:30:00 . 30m

19.5 Clarify lysates by centrifugation at 17000 x g, 4°C, 00:20:00 . 20m

Mitochondrial Enrichment: 40m

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

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

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

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

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

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

26 Discard the cytoplasmic membrane/nucleus/debris pellet.

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

28 Keep supernatant as the cytoplasmic fraction.

29 Snap-freeze the mitochondrial enriched pellet for Blue native PAGE or resuspend the pellet in mitochondria fractionation buffer with 1% Triton X-100 to keep as the mitochondrial-enriched fraction.