Protocol: **Mammalian cell culture and transfection for stable cell lines generation**

**Hina Ojha and Miratul M. K. Muqit**

Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK

**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.

1. **Materials**
	1. **Hela FI cells and plasmids:**
		1. PINK1 KO Hela Flip-In cells
		2. Doxycycline induced WT-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU43407)
		3. Doxycycline induced KI-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU46669)
		4. Doxycycline induced empty-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU45919)
		5. Doxycycline induced L532A-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU60932)
		6. Doxycycline induced L539A-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no.DU60929)
		7. Doxycycline induced L540A-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU60930)
		8. Doxycycline induced L532A L539A L540A-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU77629)
		9. Doxycycline induced R83A-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU76079)
		10. Doxycycline induced R88A-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU76082)
		11. Doxycycline induced R98A-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU76078)
		12. Doxycycline induced R83A R88A R98A-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU76087)
		13. Doxycycline induced R83E R88E R98E-PINK1-3FLAG in PINK1 KO Hela Flip-in cells (Order no. DU77573)
	2. **Consumables**
		1. Dulbecco’s modified eagle medium (DMEM) high glucose, no glutamine (GIBCO)
		2. L-glutamine 200 mM (Invitrogen), 6 mL in 500 ml media
		3. Penicillin-Streptomycin 10,000 U/mL (GIBCO); 6 mL in 500 ml media
		4. Phosphate buffered saline (Invitrogen)
		5. Hygromycin 100 mg/ml (InVivoGen), 0.5 ml in 500 ml media
		6. Blasticidin 7.5 mg/ml (InVivoGen), 1 ml in 500 ml media
		7. Zeocin 100 mg/ml (InVivoGen), 1 ml in 500 ml media
		8. Foetal bovine serum (FBS) (Sigma), 10% in media
		9. Opti-mem (Invitrogen)
		10. Doxycycline 1 mg/ml (Sigma-Aldrich), 0.02 ug/ml
		11. Lipofectamine 3000 transfection reagent (Thermofisher)
		12. 25G 1” (25mm) syringe needle (Orange)
	3. **Buffer and reagents:**
		1. **Mitochondrial fractionation buffer**: Frozen stock (final conc): 20mM HEPES pH 7.5, 3mM EDTA, 5mM Sodium β-glycerophosphate, 50mM Sodium fluoride, 5mM Sodium pyrophosphate, 250mM sucrose. Added fresh before use (final conc): 1mM Sodium orthovanadate, 1x protease inhibitor cocktail tablet (Roche).
		2. **Lysis buffer**: 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 50 mM NaF, 5 mM sodiumpyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium β-glycero-phosphate, 1 mM benzamidine, 0.1% 2-mercapto-ethanol, one mini CompleteTMprotease inhibitor cocktail tablet per 10 ml of lysis buffer and 1% Triton X-100).
	4. **Equipment**
		1. Binder CO2 Mammalian Incubator
		2. 150mm petri dishes for culturing cells
		3. VWR Micro Star 21R microcentrifuge
		4. Esco Class II biological safety cabinet
		5. Grant water bath
2. **Method**
	1. **Cell Culture**
		1. Maintain cells at 37°C in a 5% CO2 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 were from P10 to P20. The passages were never used above P25.
	2. **Maintenance of Hela FlpIn TRex Stable Cell Lines:**
		1. For HEK293 FlpIn TRex stable cell lines, use complete media supplemented with blasticidin and zeocin before recombination/transfection for stable cell line generation.
		2. Supplement with blasticidin and hygromycin B following recombination/transfection.
	3. **Generation of Stable Cell Lines:**
		1. Achieve doxycycline-induced, stable expression of exogenous protein using the FlpIn TRex system according to Invitrogen's instructions, utilizing CRISPR knock-out PINK1 KO Hela FlpIn TRex cells (Pub.No. MAN0000187) [4]. The exact steps are detailed below.
		2. Maintain Hela PINK1 null FlpIn TRex cells in blasticidin and zeocin.
		3. Give a PBS wash and transition into complete media 24 hours before recombination.
		4. Carry out recombination by co-transfecting 0.5ug integratable hygromycin-resistant pcDNA FRT/TO vector of desired PINK1/mutant with 4.5ug pOG44 expressing the Flp recombinase using lipofectamine in 100mm petri dish [5, 6].

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube 1** |  |  |  |
|  | POG44 plasmid | 4.5 ug |  |
|  | Desired DNA plasmid | 0.5 ug | Total DNA = 5ug |
|  | Lipofectamine P3000 reagent | 10 ul |  |
|  | Opti-MEM | 0.5 ml |  |
| **Tube 2** |  |  |  |
|  | Lipofectamine reagent | 7.5 ul |  |
|  | Opti-MEM | 0.5 ml |  |

* + 1. Mix the 2 tubes and keep at RT for 15 minutes.
		2. Add the transfection mix drop by drop in the plate containing Hela PINK1 null FlpIn Trex cells. Keep a plate of untransfected cells as a negative control.
		3. After 48 hours of transfection, split the cells with around 25% confluency.
		4. Once the cells are attached, add fresh complete media supplemented with blasticidin and hygromycin.
		5. 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
		6. Trypsinize surviving colonies after 3-4 weeks of selection.
		7. Expand the selected colonies, and induce protein expression with 0.02uM doxycycline.
	1. **Treatment with Mitochondrial Uncoupler:**
		1. Prepare a 50mM stock of Antimycin and 6.3mM of Oligomycin in DMSO, and store at -20°C.
		2. Uncouple mitochondria by treating with 10uM of Antimycin A and 1uM of Oligomycin for 3-6 hours, using an equivalent volume of DMSO for control conditions.
	2. **Cell Lysis and Mitochondrial Enrichment:**
		1. **Whole cell lysis**
			1. For collection keep plates with cells on ice covered with aluminium foil to provide even cool surface.
			2. Wash the cells with PBS and collect the cells with cell scraper.
			3. Collect the cells by centrifugation at 800 g for 5 min at 4°C.
			4. Add around 300ul of Lysis buffer for 100mm cell plate lysate. Resuspend the cells with lysis buffer containing 1% triton and keep them on ice for 30 min.
			5. Clarify lysates by centrifugation at 17,000g for 20 min at 4°C.
		2. **Mitochondrial Enrichment:**
			1. For collection keep plates with cells on ice covered with aluminium foil to provide even cool surface.
			2. Wash the cells with PBS and collect the cells with cell scraper.
			3. Collect the cells by centrifugation at 800 g for 5 min at 4°C.
			4. Pellet down the cells at 800 g for 5 min at 4°C. For 150 mm plate cell pellet add 300 ul of mitochondria fractionation buffer.
			5. Disrupt cell membranes using a 25-gauge needle by passing through it for 25 times on ice.
			6. Clarify lysates by centrifugation at 800g for 10 mins at 4°C.
			7. Discard the cytoplasmic membrane/nucleus/debris pellet.
			8. Isolate supernatant and centrifuge at 17,000g for 20 mins at 4°C to collect mitochondrial enriched fraction.
			9. Keep supernatant as the cytoplasmic fraction.
			10. 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.

**References**

1. Valente, E.M., et al., *Hereditary early-onset Parkinson's disease caused by mutations in.* Science, 2004. **304**(5674): p. 1158-1160.

2. Kondapalli, C., et al., *PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65.* Open Biology, 2012. **2**.

3. Themistokleous, C., et al., *Role of Autophagy Pathway in Parkinson's Disease and Related Genetic Neurological Disorders.* Journal of Molecular Biology, 2023. **435**(12).

4. Ogorman, S., D.T. Fox, and G.M. Wahl, *Recombinase-Mediated Gene Activation and Site-Specific Integration in Mammalian-Cells.* Science, 1991. **251**(4999): p. 1351-1355.

5. Broach, J.R., V.R. Guarascio, and M. Jayaram, *Recombination within the Yeast Plasmid 2-Mu Circle Is Site-Specific.* Cell, 1982. **29**(1): p. 227-234.

6. Broach, J.R. and J.B. Hicks, *Replication and Recombination Functions Associated with the Yeast Plasmid, 2-Mu Circle.* Cell, 1980. **21**(2): p. 501-508.