Sep 15, 2021

Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria

Odetta Antico[1], Alban Ordureau[2,3], Michael Stevens[1], J. Wade Harper[2], Miratul M. K. Muqit[1]

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

[2]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA;

[3]Present address: Cell Biology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

dx.doi.org/10.17504/protocols.io.bxmypk7w

m.muqit

ABSTRACT

Analysis of Parkinson's linked genes PINK1 and Parkin has uncovered a mechanism by which upon loss of mitochondrial membrane potential, Parkin E3 ubiquitin ligase activity is activated by PINK1 kinase activity, to trigger mitochondrial membrane protein ubiquitylation, leading to removal of damaged mitochondria (mitophagy). We and other groups have previously reported in vitro assays of Parkin E3 ligase activity using recombinant Parkin and PINK1 expressed in E. coli. This provided evidence of Parkin activation by PINK1 phosphorylation of Ser65 in both ubiquitin and UBL domain of Parkin. Herein, we report a reconstitution assay in which addition of recombinant Parkin to mitochondria isolated from cells after treatment by combination of Antimycin A and Oligomycin (to induce PINK1 activation on the outer mitochondrial membrane), enables robust ubiquitylation of multiple substrates at the mitochondria. This assay represents a powerful tool to study Parkin E3 ligase activity and the functional interplay between ubiquitylation and phosphorylation mediated by PINK1 and Parkin and their role in reshaping the endogenous mitochondrial proteome.

ATTACHMENTS

d58ebheux.pdf

DOI

dx.doi.org/10.17504/protocols.io.bxmypk7w

PROTOCOL CITATION

Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit 2021. Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bxmypk7w

KEYWORDS

MEFs, Mitochondria, PINK1, Parkin, Ubiquitin

CREATED

Aug 23, 2021

LAST MODIFIED

Sep 15, 2021

OWNERSHIP HISTORY

Aug 23, 2021 Urmilas

Sep 15, 2021 m.muqit

PROTOCOL INTEGER ID

52632

MATERIALS TEXT

For Mouse Embryonic fibroblast culture:

1. E13.5 mouse embryos (8–10 embryos, either sex; we used PINK1 wild-type and knockout mice) **CRITICAL!** All experiments must be conducted in accordance with the relevant institutional and governmental guidelines and regulations.

Trypsin-EDTA **Gibco - Thermo**

2. Digestion medium: 0.025% **Fisher Catalog #25300054** ; [M]**0.125 mg/ml**

DNase I **Merck Millipore**

Sigma Catalog #11284932001 in

HBSS, calcium, magnesium, no phenol red **Gibco - Thermo**

Fisher Catalog #14025050 .

3. Culturing medium:

A	B
DMEM (Gibco™ #11960-085)	
Foetal Bovine Serum (FBS) heat inactivated (Gibco™ #10500064)	20%
Penicillin-Streptomycin (Gibco™ # 15140122)	1%
LGlutamine (Gibco™ #25030024)	1%
Non-essential Amino acid (Gibco™ #11140-035)	1X
Sodium pyruvate (Gibco™ #11360-039)	1X

DMEM, high glucose, no glutamine **Thermo**

Fisher Catalog #11960085

Fetal Bovine Serum qualified heat inactivated Brazil **Gibco - Thermo**

Fisher Catalog #10500064

Penicillin-Streptomycin **Gibco - Thermo**

Fisher Catalog #15140122

L-Glutamine (200mM) **Thermo Fisher**

Scientific Catalog #25030024

MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher**

Scientific Catalog #11140035

Sodium Pyruvate (100 mM) **Thermo**

Fisher Catalog #11360039

DPBS no calcium no magnesium **Gibco - Thermo**

4. **Fischer Catalog #14190094** .

Trypan Blue solution **Sigma –**

5. **Aldrich Catalog #T8154**

Cell lines:

1. Hela ATCC (Catalog# CCL-2)

Culturing medium:

A	B
DMEM (Gibco™ #11960-085)	
Foetal Bovine Serum (FBS) (SigmaAldrich #F7524)	10%
Penicillin-Streptomycin (Gibco™ # 15140122)	1%
L-Glutamine (Gibco™ #25030024)	1%

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

DMEM, high glucose, no glutamine **Thermo Fisher Catalog #11960085**

Fetal Bovine Serum **Sigma Aldrich Catalog #F7524**

Penicillin-Streptomycin **Gibco - Thermo Fisher Catalog #15140122**

L-Glutamine (200mM) **Thermo Fisher Scientific Catalog #25030024**

For mitochondrial depolarisation and isolation:

1. Mitochondrial depolarisation: [M]**10 Micromolar (µM)** Antimycin A (Sigma-Aldrich #A8674);

[M]**1 Micromolar (µM)** Oligomycin (Sigma-Aldrich #75351) in DMSO (Sigma-Aldrich #D2650).

Antimycin A from Streptomyces sp. **Sigma – Aldrich Catalog #A8674**

Oligomycin A **Sigma – Aldrich Catalog #75351**

Dimethyl sulfoxide (DMSO) **Sigma Aldrich Catalog #D2650**

2. Hypotonic Buffer:

A	B
HEPES (pH7.8)	20 mM
KCl	5 mM
MgCl2	1.5 mM
DTT	2 mM
PMSF	1 mM
Phosphate inhibitors PhosSTOP	
Protease inhibitor cocktail	

3. 2.5X MSH (Mannitol-Sucrose-HEPES) Buffer:

Mannitol	525 mM
Sucrose	175 mM
HEPES (pH 7.8)	20 mM
EDTA	5mM
DTT	2mM
PMSF	1mM

4. 1X MSH Buffer:

A	B
Mannitol	210 mM
Sucrose	70 mM
HEPES (pH 7.8)	20 mM
EDTA	2mM
PMSF	1mM
Phosphate inhibitors PhosSTOP	
Protease inhibitor cocktail	

5. Mito Ubi Buffer (MUB):

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

A	B
Tris-HCl pH 7.5	50 mM
Sucrose	70mM
Sorbitol	210 mM
Sodium pyrophosphate	5 mM
Sodium Fluoride	50 mM
Sodium-2-glycerophosphate	10mM

DPBS no calcium no magnesium **Gibco - Thermo**

6. **Fischer Catalog #14190094**

7. Table of reagents

A	B	C
REAGENT	**COMPANY**	**CAT. NUMBER**
D (+)-SACCHAROSE (SUCROSE)	VWR	27480.36
D-SORBITOL	Merck (Sigma-Aldrich)	S1876
D-MANNITOL	Merck (Sigma-Aldrich)	M4125
EDTA DISODIUM SALT DIHYDRATE	Fisher BioReagents	BP120-500
TRIS (TROMETAMOL)	VWR	103157P
POTASSIUM CHLORIDE	VWR	26764.298
MAGNESIUM CHLORIDE HEXAHYDRATE	Merck (Sigma-Aldrich)	13152
DTT	Formedium	DTT010
HEPES	Formedium	HEPES10
2-GLYCEROPHOSPHATE DISODIUM SALT HYDRATE	Merck (Sigma-Aldrich)	G9422
PMSF	Merck (Sigma-Aldrich)	93482
SODIUM FLUORIDE	Merck (Sigma-Aldrich)	S7920
SODIUM PYROPHOSPHATE DECAHYDRATE	Merck (Sigma-Aldrich)	221368
PHOSPHATASE INHIBITORS (phosSTOP)	Merck (Sigma-Aldrich)	4906845001
COMPLETE PROTEASE INHIBITORS	Merck (Roche)	11873580001

D-( )-Sucrose AnalaR NORMAPUR® analytical reagent **VWR Chemicals Catalog #27480.360**

Mannitol **Sigma Aldrich Catalog #S1876**

D-sorbitol **Sigma Aldrich Catalog #M4125**

Ethylenediaminetetraacetic Acid Di Na Salt Dihydr. (Crystalline Powd./Electrophor.) Fisher BioReag **Fisher Scientific Catalog #BP120-500**

TRIS base **VWR Chemicals Catalog #103157P**

Potassium chloride 99.5-101.0% AnalaR NORMAPUR® Reag. Ph. Eur. analytical reagent **VWR international Ltd Catalog #26764.298**

Magnesium chloride hexahydrate **Sigma – Aldrich Catalog #13152**

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

DTT 14-DITHIOTHREITOL **Formedium Catalog #DTT010**

HEPES **Formedium Catalog #HEPES10**

β-Glycerophosphate disodium salt hydrate **Sigma – Aldrich Catalog #G9422**

Phenylmethanesulfonyl fluoride solution **Sigma – Aldrich Catalog #93482**

Sodium fluoride **Sigma – Aldrich Catalog #S7920**

Sodium pyrophosphate decahydrate **Sigma Aldrich Catalog #221368**

cOmplete ULTRA Tablets Mini EasyPack PhosStop **Sigma Aldrich Catalog #4906845001**

cOmplete™ EDTA-free Protease Inhibitor Cocktail **Roche Catalog #11873580001**

For Ubiquitylation assay:

1. Tris-Base – 103157P VWR Prepare a [M]**1 Molarity (M)** Tris-HCl pH 7.5 stock in deionised water with the pH adjusted using 37.5 % HCl.

Magnesium chloride hexahydrate **Contributed by**

2. **users Catalog #M2670-500G**

Adenosine Tri-Phosphat

3. (ATP) **Abcam Catalog #ab14730**

TRIS(2-CARBOXYETHYL)PHOSPHINE HYDROCHLORIDE **Apollo**

1. **Scientific Catalog #BIT0122**

cDNA Clone - UBE1 **MRC PPU Reagents and**

2. **Services Catalog #DU32888**

3. UbE2L3 (MRC-PPU Reagents & Services, DU3772).

cDNA Clone - Ubiquitin **MRC PPU Reagents and**

4. **Services Catalog #DU20027**

cDNA Clone - parkin **MRC PPU Reagents and**

5. **Services Catalog #DU42598**

For biochemistry

Coomassie Protein Assay Reagent **Thermo**

8. **Scientific Catalog #1856209**

NuPAGE™ LDS Sample Buffer (4X) **Invitrogen - Thermo**

9. 4X **Fisher Catalog #NP0008**

2-mercaptoethanol **Sigma**

10. **Aldrich Catalog #M6250**

PageRuler™ Prestained Protein Ladder, 10 to 180 kDa **Thermo Fisher**

11. **Scientific Catalog #26616**

Immobilon-P PVDF

12. Membrane **Merck Catalog #IPVH00010**

Amersham™ Protran® Western blotting membranes

13. nitrocellulose **Merck Catalog #GE10600041**

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

14.

NuPAGE™ 4 to 12% Bis-Tris 1.0 mm Mini Protein Gel 10-well **Invitrogen - Thermo Fisher Catalog #NP0321BOX**

,

NuPAGE™ 4 to 12% Bis-Tris 1.0 mm Midi Protein Gel 20-well **Invitrogen - Thermo Fisher Catalog #WG1402BOX**

NuPAGE™ MOPS SDS Running Buffer (20X) **Invitrogen - Thermo**

15. **Fisher Catalog #NP000102** or

NuPAGE™ MES SDS Running Buffer (20X) **Thermo Fisher Catalog #NP0002**

16. 1 X Towbin transfer buffer:

A	B
Tris	25 mM
Glycine	192 mM
Methanol	20%

17. 1X Tris Buffered-Saline (TBS): **500 Milimolar (mM)** Tris, **150 Milimolar (mM)** Sodium chloride, **7.6**, at **25 °C** .

18. 1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent (TBST).

19. 5 % Non-Fat Milk in TBST.

20. 5% Bovine serum albumin (BSA) in

Bovine Serum Albumin Fraction V **Sigma – Aldrich Catalog #10735094001**

21. Primary antibodies:

Phospho-Ubiquitin (Ser65) (E2J6T) Rabbit mAb **Cell Signaling Technology Catalog #62802**

Purified anti-Ubiquitin Antibody **BioLegend Catalog #646302**

Parkin Antibody (PRK8) **Santa Cruz Biotechnology Catalog #32282**

CISD1/mitoNEET (D5M4C) Rabbit mAb **Cell Signaling Technology Catalog #83775**

Anti-CPT1A antibody [8F6AE9] (ab128568) **Abcam Catalog #ab128568**

Cytochrome b5 Outer Mitochondrial Membrane Antibody **Novus Biologicals Catalog #NBP1-88039**

Hexokinase I (C35C4) Rabbit mAb **Cell Signaling Technology Catalog #2024**

Recombinant Anti-Mitofusin 2 antibody [NIAR164] (ab124773) **Abcam Catalog #ab124773**

VDAC (D73D12) Rabbit mAb **Cell Signaling Technology Catalog #4661**

22. Secondary Antibodies:

Goat anti-Rabbit IgG (H L) Secondary Antibody HRP **Invitrogen - Thermo Fisher Catalog #31460**

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

Rabbit anti-Mouse IgG (H L) Secondary Antibody HRP **Invitrogen - Thermo Fisher Catalog #31450**

ECL™ Western Blotting

23. Reagents **Merck Catalog #RPN2106**

SuperSignal™ West Dura Extended Duration Substrate **Thermo**

24. **Fisher Catalog #34075**

Hyperfilm™

25. ECL™ **Merck Catalog #28906837**

STOCK SOLUTION PREPARATION:

- DNaseI: Dissolve **100 mg/ml** (wt/vol) DNase in sterile double-distilled water; filter, aliquot and store at **-20 °C** . The solution is stable for 2–3 months.
- Antimycin A: Prepare **50 Milimolar (mM)** of Antimycin A in DMSO; aliquot and store at **-20 °C** .
- Oligomycin: Prepare **10 Milimolar (mM)** of Oligomycin in DMSO; aliquot and store at **-20 °C** .

EQUIPMENT:

1. Dumont #5 Forceps Biologie Inox (Fine Science Tool #11252-20).
2. Dumont #5XL Forceps Standard Inox (Fine Science Tool #11253-10).
3. Dumont #7 Fine Forceps Biologie Inox (Fine Science Tool #11274-20).
4. Student Vannas Spring Scissors Straight (Fine Science Tool #91500-09).

Scissors Iris **Fine Science**

5. **Tools Catalog #14058-09**

6. Cell Counter-DeNovix CellDropTM.
7. 37 °C water bath.
8. Laminar flow cell culture hood.
9. Cell culture incubator 5% CO2, 95% humidity HERAcell®CO2 incubator (150 L).

Microcentrifuges ventilated/refrigerated Micro Star 17 / 17R **VWR international**

10. **Ltd Catalog #521-1647**

Dounce Dura Grind® Tissue

11. Grinder **EMS Catalog #64791-07**

XCell4 SureLock™ Midi-Cell **Thermo Fisher**

12. **Scientific Catalog #WR0100**

XCell SureLock™ Mini-Cell **Thermo**

13. **Fisher Catalog #EI0001**

Mini Trans-Blot Electrophoretic Transfer Cell #1703930 **Bio-rad**

14. **Laboratories Catalog #1703930**

15.

Trans-Blot Cell With Plate Electrodes and Super Cooling Coil #1703939 **Bio-rad Laboratories Catalog #1703939**

16. ChemiDoc MP Imaging System (BIORAD).
17. ECOMAX™ X-ray Processor.
18. Eppendorf ThermoMixer – 5382000031 Eppendorf.

CONSUMABLES

1. 10cm and 15cm tissue culture Petri Dishes (

Nunc™ Cell Culture/Petri Dishes, 56.7cm2, Nunclon Delta treated, lid, vent **Thermo Fisher Catalog #172931** and

Nunc™ Cell Culture/Petri Dishes, 145 cm2, Nunclon Delta treated,lid, vent **Thermo Fisher Catalog #168381**)

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

Falcon™ Cell Strainers **Fisher**

2. **Scientific Catalog #10788201**
3. Stericups 0.22µm, 250 mL and 500 mL (

Stericup-GP Sterile Vacuum Filtration System **Fisher**

Scientific Catalog #SCGPU02RE ,

EMD Millipore™ Stericup™ Sterile Vacuum Filter Units **Fisher**

Scientific Catalog #SCGPU05RE)

4.

50 mL Stripette™ Serological Pipets Polystyrene Individually Paper/Plastic Wrapped Sterile

25/Ba **Corning Catalog #4490**

5.

25 mL Stripette™ Serological Pipets Polystyrene Individually Paper/Plastic Wrapped Sterile

25/Ba **Corning Catalog #4489**

6.

10 mL Stripette™ Serological Pipets Polystyrene Individually Paper/Plastic Wrapped Sterile

50/Ba **Corning Catalog #4488**

7.

5 mL Stripette™ Serological Pipets Polystyrene Individually Paper/Plastic Wrapped Sterile

50/Bag **Corning Catalog #4487**

15 mL conical centrifuge tube **greiner bio-**

8. **one Catalog #188271**

50 mL conical centrifuge tube **greiner bio-**

9. **one Catalog #227261**
10. Standard 1mL and 200µL Pipette tips (

PIPETTE TIPS 100- 1000 µL BLUE SUITABLE FOR EPPENDORF STERILE 60 PIECES PER RACK **greiner bio-**

one Catalog #686271

PIPETTE TIP 10 - 100 µL SUITABLE FOR EPPENDORF 96 PIECES / ST RACK **greiner bio-**

, **one Catalog #685261**)

11. Syringe filter (0.22µm. Sartorius, Item # ST16541-Q).
12. Syringes (50mL) (Terumo™# 8SS50L1).

1.5ml Safe-lock

13. tubes **Eppendorf Catalog #0030120086**

Cell Lifters **Thermo**

14. **Fisher Catalog #08100240**

PROCEDURE TO ISOLATE AND CULTURE MOUSE EMBRYONIC FIBROBLASTS-Dissection of E13.5 mouse embryos ◊TIMING 20 min

20m

1 Use sterilized instruments by autoclave or washing them with 70% (vol/vol) ethanol. Dry thoroughly if ethanol is used.

2 Soak dissection tools in 70% ethanol between embryos to prevent contamination.

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

3 In the hood. Prepare **10 cm** dishes with cold PBS. Separate embryos from uterus and placenta. Place each embryo into a single dish with cold PBS.

4 Number dishes and Eppendorf tubes for tissue collection for genotyping.

5 Euthanize the embryos by decapitation and separate the head from the body.

6 

Wash the bodies twice with PBS to minimise contamination and collect a small piece of tail for genotyping.

7 Place the body on a dish with PBS and remove the red spot (bowel) with forceps.

8 Place the body on a clean dish and mince the tissue with a spring scissors (or with a sterile scalpel blades).

Cell dissociation and plating 30m

9 

Prepare digestion medium by adding **125 µl** of DNase I (stock solution 10 mg/mL) to **10 mL** of Trypsin 0.025% (1:1 Trypsin 0.05%-HBSS).

10 

Add **5 mL** of digestion medium to the tissue and transfer in a **15 mL** falcon tube.

11  15m

Incubate at **37 °C** in a water bath for **00:15:00** .

12 

Pipette to mechanically dissociate the tissue, gentle and sequential pipetting (using **10 mL** , **5 mL** and **1 mL** pipettes) until cells are completely suspended.

Note: The number of trituration is approximative, it may vary depending on the size of the unbroken tissues.

13 Inactivate trypsin digestion by **5 mL** of culturing medium.

 5m

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

14 Centrifuge at **1200 rpm** for **00:05:00** .

15 Remove media and resuspend in **5 mL** culturing media.

16 Filter the cells through a **70 µm** filter.

Note: The cell suspension should be filtered in order to exclude any undigested tissue pieces or aggregates from the newly prepared cell suspension.

17 Take a **15 µl** aliquot, add 1:1 ratio Trypan Blu and determine the density of cells and cell viability to the cell counter.

18 Plate the 3.0×10^6 cells/well plates out on **10 cm** dishes, containing **10 mL** of pre-warmed culturing media.

19 Change media every 5 days, grow to 90% confluence and split (minimum 25% confluence to keep cells within the range to promote growth). The growth rate progressively declines when transformation occurs and the cells become immortal, at approximately after 18 passages (it can be variable).

Note: In this assay we use primary MEFs between 8-10 passages.

MITOCHONDRIAL ISOLATION FOR MEFs-Mitochondrial depolarisation ◊TIMING 4h, day of experiment 4h

20 To depolarize or uncouple mitochondrial membrane potential in MEFs, treat the cultures for **04:00:00** with a combination of [M]**10 Micromolar (µM)** Antimycin A and [M]**1 Micromolar (µM)** Oligomycin dissolved in DMSO at **37 °C** . 4h

Note: Before the experiment, MEFs were plated in 15 cm dishes and stimulated at 80-90% confluence.

MITOCHONDRIAL ISOLATION FOR MEFs-Mitochondrial isolation ◊TIMING 1-1.5h

21 Gently aspirate the medium from wells.

22 Wash twice by adding **5 mL** of warmed DPBS (room temperature) containing protease inhibitors and phosphatase inhibitors.

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

23

Place the **15 cm** dish **On ice** and add **1 mL** of Hypotonic Buffer. Carefully scrape the cells and collect the cells in a **15 mL** microcentrifuge tube. Add **2 mL** of Hypotonic Buffer in each tube (for a total of **3 mL**).

24 15m

Stand **On ice** for **00:15:00** in the cold room.

25

Homogenise cells using a stainless steel Dounce homogeniser with 45 strokes.

Note: Check cell disruption with light microscope, 80-90 % cells should be disrupted.

26

Add to the disrupted cells 2.5X MSH buffer and mix.

Note: Mixing 2.5X MSH volume to the initial volume of hypotonic buffer will give 1X MSH.

27 10m

Centrifuge the homogenate at **700 x g** in a refrigerated centrifuge for **00:10:00** , to remove cell debris and nuclei.

28 10m

Transfer supernatant into a new **15 mL** tube and centrifuge at **700 x g** x **00:10:00** at **4 °C** to remove residual nuclei and cell debris.

29 10m

Centrifuge at **9000 x g** x **00:10:00** at **4 °C** to pellet mitochondria.

30 10m

Resuspend mitochondria in **1 mL** of 1X MSH buffer and centrifuge at **9000 x g** x **00:10:00** at **4 °C** to pellet mitochondria. Repeat twice to remove any cytosolic proteins.

31 10m

Centrifuge at **9000 x g** x **00:10:00** at **4 °C** to pellet mitochondria and resuspend in **150 µl** of MUB Buffer.

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

32 Protein quantification: take a small aliquot of mitochondria (10 µL), add 1% Triton, vortex and estimate protein concentration by using the Coomassie Protein Assay.

Usually from a 15 cm dish of MEFs at 90% confluence it is possible to isolate 200µg of crude mitochondria.

MITOCHONDRIAL ISOLATION FOR HeLa-Mitochondrial depolarisation ◊TIMING 2h, day of experiment 2h

33 2h
To depolarize or uncouple mitochondrial membrane potential in MEFs, treat the cultures for **02:00:00** with a combination of **10 Micromolar (µM)** Antimycin A and **1 Micromolar (µM)** Oligomycin dissolved in DMSO at **37 °C** .

Note: Before the experiment Hela were plated in 15 cm dishes and stimulated to 80- 90% confluence.

MITOCHONDRIAL ISOLATION FOR HeLa-Mitochondrial isolation ◊TIMING 1-1.5h 2h

34 Gently aspirate the medium from wells.

35 

Wash twice by adding **5 mL** of warmed DPBS (**Room temperature**) containing protease inhibitors and phosphatase inhibitors.

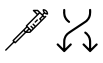
36 

Place the **15 cm** **On ice** and add **1 mL** of Hypotonic Buffer. Carefully scrape the cells and collect the cells in a **15 mL** microcentrifuge tube. Add **2 mL** of Hypotonic Buffer in each tube (for a total of **3 mL**).

37 15m
Stand **On ice** for **00:15:00** in the cold room.

38 Homogenise cells using a stainless steel Dounce homogeniser with 25 strokes.

Note: Check cell disruption with light microscope, 80-90 % cell should be disrupted.

39 

Add to the disrupted cells 2.5X MSH buffer and mix.

Note: Mixing 2.5X MSH volume to the initial volume of hypotonic buffer will give 1X MSH.

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

40 10m

Centrifuge the homogenate at **700 x g** in a refrigerated centrifuge for **00:10:00** , to remove cell debris and nuclei.

41 10m

Transfer supernatant into a new **15 mL** tube and centrifuge at **700 x g** x **00:10:00** at **4 °C** to remove residual nuclei and cell debris.

42 10m

Centrifuge at **9000 x g** x **00:10:00** at **4 °C** to pellet mitochondria.

43 10m

Resuspend mitochondria in **1 mL** 1X MSH buffer and centrifuge at **9000 x g** x **00:10:00** at **4 °C** to pellet mitochondria. Repeat twice to remove any cytosolic proteins.

44 10m

Centrifuge at **9000 x g** x **00:10:00** at **4 °C** to pellet mitochondria and resuspend in **150 µl** - **250 µl** of MUB Buffer.

45 Protein quantification: take a small aliquot of mitochondria (**10 µl**), add 1% Triton, vortex and estimate protein concentration by using the Coomassie Protein Assay.

Usually from one 15 cm dish of HeLa at 80-90% confluence it is possible to isolate 600-800µg of crude mitochondria.

MITOCHONDRIAL ISOLATION FOR HeLa-UBIQUITYLATION ASSAY ◊TIMING 2.5h-3h

46 Resuspend isolated mitochondria in MUB buffer at concentration ~ **1 mg/ml** , in order to use a volume of mitochondria < 10% of reaction volume.

47 Use **5 µg** of mitochondria for a total volume reaction of **50 µl** .

48 Defrost proteins **0 °C** and prepare a master mix considering that for one single reaction it is required **1 Micromolar (µM)** Parkin, **0.1 Micromolar (µM)** His-UbE1, **1 Micromolar (µM)** UBE2L3 and **30 Micromolar (µM)** Ubiquitin (as negative control prepare a master mixer without Parkin).

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

49 Prepare reaction buffer with [M]**50 Milimolar (mM)** Tris pH**7.5**, [M]**5 Milimolar (mM)** MgCl2 and [M]**0.5 Milimolar (mM)** TCEP.

50

Combine reaction buffer, master mix of proteins and mitochondria.

51

Aliquot in order to distribute **50 µl** in **1.5 mL** Eppendorf tube and start the ubiquitylation reaction by adding [M]**2 Milimolar (mM)** ATP in each Eppendorf tube.

52 3h 30m

Place the eppendorf tubes in a thermomixer and incubate the reaction at **30 °C**, shaking for **02:00:00** (MEFs) or **01:30:00** (HeLa) at **1000 rpm**.

53 Stop the reaction by the addition of 4X LDS loading buffer containing 10% of 2- mercaptoethanol.

MITOCHONDRIAL ISOLATION FOR HeLa-Immunoblotting of ◊TIMING 5h-2d 4h 53m

54 3m

Boil the samples for **00:03:00** at **97 °C**.

55 Analyse samples by running **20 µl** of reaction on Nu-page Bis-Tris 4-12% gels for a better resolution of ubiquitin chains, at 120 V for ~2h. Use MES SDS Running Buffer or MOPS SDS Running Buffer according to the size of protein to be analysed.

For phospho-parkin and Parkin blots, it is recommended to dilute the final reaction 1 in 25 with 1X LDS containing 2.5 % 2- mercaptoethanol.

56 1h 30m

Transfer gel on PVDF membrane for phospho-ubiquitin and ubiquitin signal and nitrocellulose membrane for phospho-Parkin and Parkin signal. Transfer in Towbin buffer at 80 V for **01:30:00** **On ice** or in cold room (for HK1 it is recommended to transfer at 90 V for 1.5h).

Note: Prepare only 1 membrane per transfer tank –avoid multiple membranes for transfer in same tank as this reduces ubiquitin transfer.

57 1h

Incubate membrane with blocking buffer 5% milk in 0.1% TBS-Tween for **01:00:00** at **Room temperature**

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w

58 1h

Remove blocking buffer, if primary antibodies are in 5% BSA, rinse twice with 0.1% TBS-Tween to remove any traces of milk, add primary antibodies and incubate **Overnight** at **4 °C** .

Note: Prepare phospho-Ubiquitin Antibody (1:2000), Ubiquitin Antibody (1:1000), CISD1 Antibody (1:1000), CPT1α Antibody (1:1000), CYB5B Antibody (1:1000), HK1 Antibody (1:1000), MFN2 Antibody (1:1000), VDAC Antibody (1:1000) and Parkin Antibody (1:1000) in 5% BSA (TBS-Tween). Prepare phospho-Parkin Antibody (1:2000) in 5% milk (TBS-Tween). To avoid non-specific signal, it is recommended to preincubate phospho-Parkin antibody with a membrane for 2 days before using it.

59 10m

Remove primary antibody and wash 3 times with 0.1%TBS-Tween for **00:10:00** .

60 1h

Add secondary antibodies, HRP-conjugate for **01:00:00** at **Room temperature** diluted 1:5000 in 1% BSA (0.1% TBS-Tween). Use 1:10000 dilution in 1% BSA for Parkin antibody and 1:10000 dilution in 5% milk for and phospho-Parkin antibody.

61 10m

Remove secondary antibody and wash 3 times with 0.1%TBS-Tween for **00:10:00** .

62 Develop signal using ECL western Blotting reagents and analysing with Chemidoc.

Note: Depending on signal, film can be best for sensitivity. To improve detection of HK1 and VDAC ubiquitylation, it is recommended to develop signal using Super signal West Dura reagents.

Citation: Odetta Antico, Alban Ordureau, Michael Stevens, J. Wade Harper, Miratul M. K. Muqit (09/15/2021). Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria. https://dx.doi.org/10.17504/protocols.io.bxmypk7w