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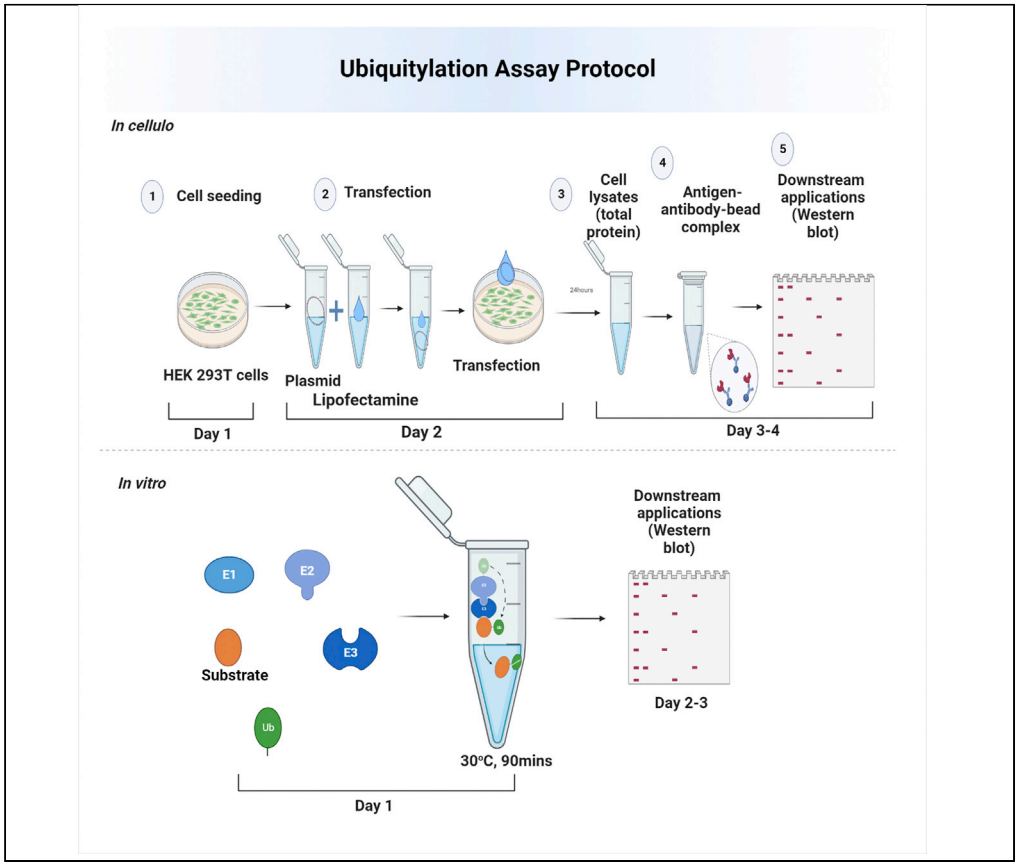
Protocol to detect *in vitro* and in cell ubiquitylation of mitochondrial DNA polymerase gamma by mitochondrial E3 ligase MITOL



Mitochondrial polymerase gamma (PolγA) is the only replicative polymerase in mitochondria. To determine PolγA ubiquitylation in cells, Flag-PolγA and MITOL are overexpressed, and subsequently the immunoprecipitated Flag-PolγA is checked for ubiquitylation. Alternately, *in vitro* synthesized PolγA and MITOL are used to determine whether PolγA is ubiquitylated. Either anti-ubiquitin or anti-Flag antibody is used to detect the ubiquitylated product. Thus, we provide a detailed, reliable, highly reproducible protocol for detecting ubiquitylation of PolγA by MITOL, both in cells and *in vitro*.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

PolγA ubiquitylation by mitochondrial E3 ligase, MITOL is detected *in vitro* and in cells

Immunoprecipitated PolγA from cells or *in vitro* synthesized PolγA are used as substrate

Ubiquitylated PolγA is detected as a smear above its native molecular weight, 150 kDa

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Protocol to detect *in vitro* and in cell ubiquitylation of mitochondrial DNA polymerase gamma by mitochondrial E3 ligase MITOL

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SUMMARY

Mitochondrial polymerase gamma (PolγA) is the only replicative polymerase in mitochondria. To determine PolγA ubiquitylation in cells, Flag-PolγA and MITOL are overexpressed, and subsequently the immunoprecipitated Flag-PolγA is checked for ubiquitylation. Alternately, *in vitro* synthesized PolγA and MITOL are used to determine whether PolγA is ubiquitylated. Either anti-ubiquitin or anti-Flag antibody is used to detect the ubiquitylated product. Thus, we provide a detailed, reliable, highly reproducible protocol for detecting ubiquitylation of PolγA by MITOL, both in cells and *in vitro*.

For complete details on the use and execution of this protocol, please refer to Hussain et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps to check PolγA ubiquitylation, both in vitro and in cells. It is essential to prepare the wildtype versions of active recombinant E3 ligase MITOL (also known as MARCH5 or RNF153) and the target protein Polymerase gamma A (PolγA) for *in vitro* ubiquitylation. A catalytically-dead, recombinant version of the MITOL (called MITOL CD), detailed in (Hussain et al., 2021), needs to be generated to understand the E3 ligase specificity of the ubiquitylation assay. As detailed in (Hussain et al., 2021), wildtype PolγA is ubiquitylated by MITOL at position Lysine 1060 in the protein sequence. Hence a recombinant PolγA mutant where Lysine 1060 is changed to arginine (PolγA K1060R) also needs to be generated to prove that MITOL ubiquitylates PolγA specifically at Lysine 1060. Also, generate high-quality supercoiled plasmids which will be used for transfection in HEK293T cells and for in vitro protein synthesis (using *in vitro* transcription and translation or TNT method) need to be generated prior to starting.

The *in vitro* ubiquitylation reaction was carried out using commercially available recombinant proteins (E1, E2 and ubiquitin), lab purified recombinant E3 ligase (MITOL) and target protein (PolγA), followed by Western blotting using Ubiquitin/PolγA specific antibodies. For determining the ubiquitylation in cells, co-transfection of myc-tagged MITOL and Flag-tagged PolγA expressing plasmids in HEK293T cells was carried out, followed by immunoprecipitation using anti-Flag M2 affinity gel. To detect PolγA ubiquitylation in cells, Western blot analysis was carried out with either anti-Flag or anti-PolγA or anti-ubiquitin antibodies. While in this protocol HEK293T cells have been used, the same process can be tried out in other cell types as well after due standardization.

Preparation of high-quality plasmids

Timing: 3 days

1. Plasmids carrying the sequences coding for the His-tagged Ubiquitin construct, Flag-tagged PolγA (pcDNA3.1 Flag-PolγA) and myc-tagged MITOL WT/CD (detailed in the key resources table) are to be transformed in *E. coli* DH5α competent cells.

The plasmids were isolated from DH5α cells cultured in 100 mL of Luria-Broth (LB). DH5α cells were grown in presence of the selection marker Ampicillin (final concentration 100 μg/mL) at 37°C overnight (16 h) using a QIAGEN Midi prep kit (details in the key resources table). Plasmids were eluted in 100 μL elution buffer provided in the kit. The concentration of plasmids can be analyzed using a spectrophotometer. Plasmid yield usually range from 100-300 μg per sample. Plasmids are to be aliquoted into small volumes (typically 25 μL) and stored at −20°C. Multiple freeze-thaw cycles of the stock plasmids are to be avoided to maintain their supercoiled form.

Note: Instead of DH5α cells, it is also possible to use Max efficiency STBL2 or One Shot STBL3 cells for plasmid propagation. The number of freeze thaw cycles for plasmid prep should not exceed six.

Preparation of PolγA for *in vitro* ubiquitylation

Timing: 2 h

2. Preparation of *in vitro* transcribed and translated PolγA.
   a. T7 coupled transcription/translation (TNT) reaction mix is stored at −80°C as 20 μL aliquots. Thaw one aliquot of the TNT reaction mix and 1 mM methionine stocks present in the kit on ice for 5–10 min.
   b. Add 1 μg of pcDNA3.1 Flag-PolγA or pcDNA3.1 Flag-PolγA K1060R and 1 μL of 1 mM methionine to the TNT reaction aliquot (20 μL) and make up the total reaction volume to 25 μL using nuclease-free water. Mix by vortexing gently or slight tapping followed by a quickly spinning down all the ingredients in the Eppendorf tube in a benchtop or tabletop centrifuge for 10 s (at 100 × *g*).
   c. Incubate the reaction mix at 30°C for 90 min in a circulatory water bath or thermomixer with mild shaking (at 100–200 rpm).
   d. Stop the reaction by keeping the sample on ice for 10 min.
   e. To check whether unlabeled PolγA (WT or K1060R) has been prepared, 1 μL from the reaction mix has to be electrophoresed on a 10% SDS-PAGE gel, followed by Western blot analysis with anti-PolγA antibody. Expected molecular weight of PolγA is 140 kDa.

Note: In case unequal amounts of the two proteins (PolγA WT and PolγA K1060R) were synthesized in the TNT reactions, then a gradient of each protein (0.2 μL–1 μL) has to be run on SDS-PAGE and Western blot analysis has to be repeated. Each blot has to be scanned in a densitometer and process repeated till the amounts of the two proteins are equalized.

Preparation of recombinant proteins use for *in vitro* ubiquitylation of PolγA

Timing: 4 days

Note: The recombinant proteins E1 and E2 ubiquitin used for *in vitro* ubiquitylation assay are commercially available (see key resources table for details). PolγA and MITOL were expressed in *E. coli* and purified in lab.

3. Preparation of GST-tagged recombinant MITOL (wildtype: WT and catalytically dead: CD).
   a. Transform 10 ng of pGEXT4T-1 MITOL (WT and CD) in *E.coli* BL21 codon plus RP cells. Post transformation, a single cell colony was picked from the plate and inoculated in 5 mL LB media (with Ampicillin 100 μg/mL final concentration) overnight at 37°C with shaking (at 200 rpm). An inoculum (0.05%) was added to 100 mL of LB media. Cells were grown in presence of antibiotic (final concentration of Ampicillin 100 μg/mL) at 37°C with shaking (200 rpm) up to a cell density of 0.5 (OD to be taken at 600 nm).

   Note: While induction was checked only with BL21 codon plus RP cells, other cell types may also be used after standardization.

   b. Induce the protein expression by adding isopropyl-thio-β-galactoside (IPTG) at a final concentration of 1 mM and incubating the culture at 200 rpm at 18°C for 6 h.
   c. Pellet down the cultured cells by centrifugation at 226 × *g* for 10 min at 4°C. Discard the supernatant.
   d. Resuspend the pellet by pipetting in 10 mL ice-cold 1× PBS (pH 7.4) (from 10× stock) supplemented with 1× protease inhibitor cocktail (from the 100× stock as recommended by the manufacturer), 1 mM PMSF (from freshly prepared 100 mM stock) and 1 mM DTT (from 1 M stock which must not be frozen and thawed more than 5 times and hence stored in small aliquots).
   e. Transfer resuspended bacterial cells into a fresh 50 mL high-clarity polypropylene tube and lyse bacterial cells by a minimum of 5 sonication cycles (30 s ON and 30 s OFF with 20% power). Total lysate volume which should be obtained post-sonication will be approximately 10 mL.

   Note: During sonication, keep the samples on ice when the machine is in both ON and OFF conditions. Every effort should be made to ensure that the samples are not affected by the heat generated in the sonication step. Post sonication, samples should look translucent or clear. If the samples still look turbid or viscous, increase cycle numbers till the sample looks clear or loses viscosity (typically 10 cycles should be able to get the clear or translucent look of the supernatant). While the sonicator used was Bandelin Sonoplus HD2070 using a 2 mm microtip, other types of sonicators using either 2 mm or 3 mm micro-tip can also be used. (The time and power used in the sonication step may vary based on the make of the sonicator).

   f. Post-sonication add 1 mL of Triton-X 100 prepared in 1× PBS to 9 mL of the bacterial cell lysates (final concentration of Triton X-100 should be 0.2%) and keep the samples at 4°C in an rotary mixer for 30 min. This step helps to achieve maximum protein yield by disrupting bacterial cells.
   g. Centrifuge the above cell suspension at 17,000 × *g* for 30 min at 4°C and transfer the clear supernatant containing desired proteins to a fresh tube.

   Note: Equilibrate the Glutathione Sepharose resin in GST buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 15% glycerol, 0.5% Nonidet-40). Add 1 mL of GST buffer to 100 μL of Glutathione Sepharose resin and spin at 145 × *g* at 4°C for 5 min. Pipette out the supernatant very slowly taking utmost care that the gel pellet is not disturbed. Repeat this step twice.

   h. Add 100 μL equilibrated Glutathione Sepharose resin to the supernatant and incubate at 4°C on rotary mixer for 1 h.
   i. Wash the beads three times with GST buffer at 145 × *g* at 4°C for 5 min each. In each wash, pipette out the supernatant and resuspend the beads in fresh 1 mL GST buffer.
   j. Load the washed GST resin onto the Poly-prep chromatography column at 4°C and elute protein using a stepwise gradient of reduced glutathione (0.5 mM, 1 mM, 2.5 mM, 5 mM) prepared in GST buffer. The volume of each elute will be 50 μL. Collect the eluates in 3–4 fractions.

k. Take 5 μL from each eluated fraction and check on a Coomassie-stained SDS-PAGE gel.
l. Pool the fractions containing pure proteins and perform dialysis in a freshly prepared 500 mL dialysis buffer (50 mM Tris-HCl pH-7.5, 150 mM NaCl, 20% glycerol and 1 mM DTT) at 4°C for a total of 6 h. After 3 h replace the old buffer with freshly prepared dialysis buffer.

Note: The dialysis membrane used (detailed in key resources table) has a cut off of 14 kDa. The dialysis membrane should be chosen based on the molecular weight of the protein which is desired to be purified. The method used for preparation of dialysis membrane is found in https://www.med.upenn.edu/robertsonlab/assets/user-content/documents/Preparation%20of%20Dialysis%20Tubing.pdf.

m. Store the dialyzed purified recombinant proteins in multiple aliquots (aliquot size 5–10 μL) at −80°C until further use (can be stored for approximately 6 months).

Transfection for detection of ubiquitylation in cells

Timing: 2–3 days

4. HEK293T cells seeding.

Note: Every effort should be made to keep HEK293T cells in optimal growth conditions. Cells should be regularly visualized by microscopy and checked for proper morphology. There should not be floating or dead cells. Always split the cells around 80% confluency. Never let the cells achieve near 100% confluency while sub-culturing them.

a. Aspirate the media and wash the cells with 10 mL sterile 1× PBS.
b. Aspirate the 1× PBS and add 1 mL Trypsin EDTA solution (0.25%) to detach the cells. Incubate cells at 37°C for 2 min.

Note: Observe the cells periodically under a microscope to make sure that all cells are detached and floating. HEK293T cells self-detach upon trypsin treatment. Hence there is no need for hard detachment by tapping the flasks.

c. Add 2 mL of complete media (advanced DMEM medium, supplemented with 10% Fetal bovine serum, 2 mM L-Glutamine solution and 1% Pen-Strep-Amphotericin B solution) to stop trypsin activity. Collect the cell suspension in a 15 mL tube and centrifuge at 145 × *g* for 5 min at room temperature.
d. Carefully remove supernatant and resuspend the cell pellet using a 1 mL or 5 mL pipette in 3 mL complete media.
e. Measure cell count using a hemocytometer and seed approximately 0.1 million cells to each well of a 6 well cluster plate.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-PolγA (Rabbit polyclonal) (used at 1:2000 dilution)	Santa Cruz Biotechnology	Cat# sc-48815; RRID: AB_2166864
Anti-PolγA (goat polyclonal) (used at 1:1000 dilution)	Santa Cruz Biotechnology	Cat# sc-5930; RRID: AB_2166868

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-PolγA (Rabbit polyclonal) (used at 1:2000 dilution)	Novus Biologicals	Cat# NBP1-33633 RRID: AB_2166860
Anti-Myc-tag (Rabbit monoclonal) (used at 1:1000 dilution)	Cell Signaling Technology	Cat#2278; RRID: AB_10693332
Anti-hsp60 (Rabbit polyclonal) (used at 1:2500 dilution)	Abcam	Cat# ab87085; RRID: AB_10672924
Anti-Flag (Mouse monoclonal) (used at 1:1000 dilution)	Merck	Cat# F1804; RRID: AB_262044
Anti-Ubiquitin (P4D1) (Mouse monoclonal) (used at 1:1000 dilution)	Santa Cruz Biotechnology	Cat# sc-8017; RRID: AB_628423
Anti-Flag M2 affinity gel (used at 2.5 μL/ immunoprecipitation reaction)	Merck	Cat# F2220
Bacterial and virus strains		
BL21-CodonPlus-RP	Agilent	Cat# 230250
DH5α	Thermo Fisher Scientific	Cat# 18265017
Biological samples		
pA Puro MITOL WT-myc or Myc MITOL WT	Shigeru Yanagi (Tokyo University of Pharmacy and Life Sciences, Japan)	N/A
pA Puro MITOL CD-myc or Myc MITOL CD	PMID: 33657094	N/A
pGEX4T-1 MITOL WT or GST MITOL WT	PMID: 33657094	N/A
pGEX4T-1 MITOL CD or GST MITOL CD	PMID: 33657094	N/A
His-Ub	Akhil Banerjea, (National Institute of Immunology, India)	N/A
pcDNA3.1 hygro(+)-Flag-PolγA WT	PMID: 33657094	N/A
pcDNA3.1 hygro(+)-Flag-PolγA K1060R	PMID: 33657094	N/A
Chemicals, peptides, and recombinant proteins		
Ubiquitin	Enzo	Cat# BML-UW8795-0005
Ubiquitin activating enzyme E1 Ube 1 (human)	Enzo	Cat# BML-UW9410-0050
UbcH5a (E2) (human)	Enzo	Cat# BML-UW9050-0100
GST MITOL Wildtype (WT)	PMID: 33657094	N/A
GST MITOL Catalytic Dead (CD)	PMID: 33657094	N/A
Other		
T7 Quick coupled Transcription/ Translation (TNT) system	Promega	Cat# L2080
Lipofectamine 2000	Thermo Fisher Scientific	Cat# 11668019
Complete Protease Cocktail inhibitor	Roche	Cat# 11697498001
Immobilon Western Chemiluminescent HRP substrate	Merck	Cat# WBKLS0500
Plasmid Midi Kit	QIAGEN	Cat# 12145
Nitrocellulose membrane	Bio-Rad Laboratories	Cat#1620115
IPTG	Merck	Cat# I6758
PMSF	Merck	Cat# P7626
DTT	Merck	Cat# D0632
Triton-X-100	Merck	Cat# T9284
Bovine Serum Albumin	New England Biolabs	Cat# B9000S
Ampicillin	Merck	Cat# A9518
Glutathione S-Sepharose High Performance	Cytiva	Cat# 17-5279-02
Poly-prep chromatography column	Bio-Rad	Cat# 731-1550
Reduced glutathione	Merck	Cat# G6529
Coomassie blue G	Merck	Cat# B0770
Dialysis tubing cellulose membrane	Merck	Cat# D9277
Advanced DMEM	Thermo Fisher Scientific	Cat# 12491-023
Optimem reduced serum medium	Thermo Fisher Scientific	Cat# 31985-088
Trypsin (2.5%)	Thermo Fisher Scientific	Cat#15090-046
L-Glutamine	Biological Industries	Cat# 03-020-1B
Penicillin-Streptomycin Amphotericin B Solution	Biological Industries	Cat# 03-033-1B
Water	Merck	Cat# W4502

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Potassium chloride	Merck	Cat# P9333
Magnesium chloride	Merck	Cat# M8266
Nonidet P-40 substitute	Merck	Cat# 74385
Trizma base	Merck	Cat# T1503
Sodium chloride	Merck	Cat# S9888
EDTA	Merck	Cat# E3889
EGTA	Merck	Cat# E3889
Glycerol	Merck	Cat# G2025
ATP	Merck	Cat# A9187
Disodium Hydrogen Phosphate	Merck	Cat# S7907
Sodium dihydrogen phosphate	Merck	Cat# S8282
Sodium dodecyl sulfate	Merck	Cat# L3771
Bromophenol Blue	Bio-Rad	Cat# 1610404
Bovine Serum Albumin	Merck	Cat# A6003
MG132	UBPBio	Cat# F1101
PMSF	Merck	Cat# P7626
Tween 20	Merck	Cat# P7949
Prestained SDS-PAGE Standards, broad range	Bio-Rad	Cat# 1610374
Quick Start™ Bradford 1× Dye Reagent	Bio-Rad	Cat# 5000205
Sepharose CL6B	Merck	Cat# CL6B200
Skim Milk	Difco	Cat# 232100
Peroxidase conjugated Donkey anti-Mouse IgG	Jackson ImmunoResearch	Cat# 715-035-150
Peroxidase conjugated Donkey anti-Rabbit IgG	Jackson ImmunoResearch	Cat# 711-035-152
Fetal bovine serum (FBS)	Thermo Fisher Scientific	Cat# 10082147
Experimental models: Cell lines		
HEK293T	Present in the lab of corresponding author	ATCC Cat# CRL-3216

MATERIALS AND EQUIPMENT

Key assay buffers and solutions

In vitro transcription and translation reaction.

For protein synthesis		
Reagent	Final concentration	Volume
T7 quick coupled translation/transcription reaction (TNT) mix	n/a	20 μL
pcDNA3.1 Flag-PolγA or pcDNA3.1 Flag-PolγA K1060R (Stock concentration of plasmids 1 μg/μL)	1 μg (total amount)	1 μL
1 mM Methionine (present as part of the TNT kit)	0.04 mM	1 μL
Nuclease-free water	for volume makeup	3 μL
Total	n/a	**25 μL**

Note: Reaction is carried out at 30°C. The reaction product is aliquoted (5 μL/aliquot) and can be stored at −80°C for up to two months until use.

GST buffer		
Reagent	Final concentration	Amount
1 M Tris-HCL pH 7.5	50 mM	2.5 mL
2 M KCL	100 mM	2.5 mL
1 M $MgCl_2$	10 mM	0.5 mL
1 M DTT	1 mM	50 μL
100% Glycerol	15%	7.5 mL
100% Nonidet P-40	0.5%	0.25 mL
Water	For volume make-up	36.7 mL
Total	n/a	**50 mL**

Note: Store at 4°C for up to 1 month. Add 1× Protease inhibitor cocktail (from 100× stock as recommended by the manufacturer) and 1 mM PMSF (from freshly prepared stock 100 mM PMSF) just before use.

M2 lysis buffer		
Reagent	Final concentration	Volume
1 M Tris-HCL pH 7.4	50 mM	2.5 mL
5 M NaCl	150 mM	1.5 mL
0.5 M EDTA	0.5 mM	50 µL
0.5 M EGTA	0.5 mM	50 µL
100% Glycerol	10%	1 mL
100% TritonX100	1%	500 µL
Water	For volume make-up	44.4 mL
Total	n/a	**50 mL**

Note: Store at 4°C for up to 1 month. Add 1× Protease inhibitor cocktail (from 100× stock) and 1 mM PMSF (from freshly prepared stock 100 mM PMSF) just before use.

***In vitro* ubiquitylation assay buffer**			
Reagent	Stock concentration (10× buffer)	Final concentration (1× buffer)	Volume for 10× stock buffer (µL)
1 M Tris-HCL pH 7.5	400 mM	40 mM	20 µL
1 M $MgCl_2$	50 mM	5 mM	2.5 µL
100 mM ATP	20 mM	2 mM	10 µL
Water	For volume make-up	For volume make-up	17.5 µL
Total	n/a	n/a	50 µL

Note: Prepare this buffer fresh every time.

10× PBS buffer		
Reagent	Component concentration (10× buffer)	For 1 ltr of 10× PBS buffer (dissolve below mentioned salts)
NaCl	1.37 M	80 g
KCl	27 mM	2 g
Na2HPO4	100 mM	17.8 g
KH2PO4	18 mM	2.4 g
Water	n/a	**Make up volume up to 1 Liter**
Total	n/a	**1,000 mL**

Note: Adjust the pH of 10× PBS Buffer to 7.4 with hydrochloric acid or sodium hydroxide. Can be stored up to 1 month.

Dialysis Buffer			
Reagent	Stock concentration	Final concentration	Volume of reagents for 1 liter dialysis buffer
Tris-Cl (pH 7.5)	1 M	50 mM	50 mL
NaCl	3 M	150 mM	50 mL

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Reagent	Stock concentration	Final concentration	Volume of reagents for 1 liter dialysis buffer
Glycerol	100%	20%	200 mL
DTT	1 M	1 mM	1 mL
Water	n/a	n/a	**Make up volume up to 1 Liter**
Total	n/a	n/a	**1,000 mL**

Note: Adjust the pH of 10× PBS Buffer to 7.4 with hydrochloric acid or sodium hydroxide. Stored in 4°C for 6 months.

2× gel loading dye

Reagent	Stock concentration	Final concentration	Volume of reagents for 10 mL of the 2× gel loading dye
Tris-Cl (pH 6.8)	1 M	50 mM	500 µL
SDS	Solid	2%	200 mg
Glycerol	100%	10%	1 mL
EDTA, pH8.0	0.5 mM	12.5 mM	250 µL
DTT	1 M	200 mM	2 mL
Bromophenol blue	Solid	0.02%	2 mg
Water	n/a	n/a	**Make up volume to 10 mL**
Total	n/a	n/a	**10 mL**

Stored in −20°C up to 6 months in small aliquots (maximum volume 200 µL). It is preferable that DTT is added immediately before use. If DTT is not added, the dye can be stored at 4°C.

STEP-BY-STEP METHOD DETAILS

In vitro ubiquitylation assay of PolγA

Timing: 3 days

The following protocol uses Ube1 (as the E1), UbcH5a (as the E2), MITOL (as the E3) and PolγA (as the substrate) in presence of ATP (in concentration mentioned above in the *in vitro* ubiquitylation assay buffer) and ubiquitin.

1. It is essential to determine the exact concentrations of lab purified recombinant proteins MITOL (WT and CD). For this purpose, the two lab purified recombinant proteins were electrophoresed in SDS-PAGE. In the same SDS-PAGE gel, known amounts of Bovine Serum Albumin (BSA) protein were also electrophoresed. The entire gel was stained by Coomassie. The exact concentrations of MITOL WT and MITOL CD were determined by comparing the relative intensity of their bands with the bands of the BSA gradient using a densitometer. Instead of BSA any other easily available marker proteins can also be used.

   Note: While a range of BSA (1 µg–5 µg) as standard was used to measure protein concentrations of MITOL WT and MITOL CD, this range of BSA standards may have to be varied depending on the concentration of the purified proteins.

2. Perform *in vitro* ubiquitylation assay.
   a. Prepare *in vitro* ubiquitylation assay mix as follows:

Reagent	Final concentration	Volume
In vitro ubiquitylation buffer (10×)	1×	2 µL
Ube1 (E1) (stock 847.46 nM)	42.373 nM	1 µL
UbcH5a (E2) (stock 17.65 µM)	0.8825 µM	1 µL

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Reagent	Final concentration	Volume
Ubiquitin (stock 2 μM)	0.1 μM	1 μL
MITOL (E3) WT/CD (898 nM)	76 nM	1.7 μL
TNT generated PolγA WT or PolγA K1060R (substrate protein)	n/a	0.2–1 μL
Water	n/a	For final volume make-up
Total	n/a	**20 μL**

Note: Advantage of using *In vitro* transcription cum translation system (TNT) is that it mimics eukaryotic transcription and translation with post-translational modifications on target protein. If the ubqituitylation of target protein is post translational modification dependent, then it is preferable to use TNT produced target proteins. Alternatively, *E.coli* purified recombinant proteins can also be used.

b. Incubate the mix at 30°C for 2 h with mild shaking in a temperature-controlled mixer or circulating water bath.
c. Add 20 μL of 2×SDS loading dye to stop the reaction. Mix well and boil the sample in boiling water or heat block (at 100°C) for 10 min.
d. Briefly spin down the samples and separate them on 10% SDS PAGE gel.
e. Analyze ubiquitylation of substrate protein by Western blot using an antibody against Ubiquitin. To determine that under all conditions equal amounts of PolγA (WT or K1060R) was used an anti-PolγA blot also needs to be done.

Note: 1. If PolγA is ubiquitylated, it will be detected as a smear above 150 kDa. Equal levels of the substrate protein (i.e., PolγA) should also be visualized in western blot (Figure 1).

Note: 2. The above *in vitro* ubiquitylation protocol has used recombinant wildtype ubiquitin. To determine the ubiquitin linkages, specific commercially available antibodies or affimers are to be used. Alternately commercially available recombinant ubiquitin mutants can also be used.

In cell ubiquitylation assay of PolγA

Timing: 3 days

The following protocol is a detailed procedure to analyze the *in cellulo* ubiquitylation of PolγA using the transient transfection method in HEK293T cells. Following steps are performed in laminar air flow hood.

3. Plasmid transfection.
   a. Seed 0.1 × 10^6 HEK293T cells in a 6 well cluster plate 24 h before transfection. Grow the cells in a 37°C incubator having 5% carbon dioxide.
   b. Next day the cells should be in 60%–70% confluent. Pre-warm Opti-MEM media; keep the plasmids and lipofectamine reagents ready for transfection.
   c. Add 250 μL Opti-MEM in Tube A.
   d. Add 1 μg Flag-PolγA, 1 μg Myc-MITOL WT and MITOL CD and 100 ng His-Ubiquitin plasmids to Tube A and mix well.
   e. Add 250 μL Opti-MEM to Tube B.
   f. Add a 1:3 ratio of Lipofectamine 2000 to Tube B and mix well and incubate the reaction at room temperature for 5 min (For example, if 2.1 μg of total plasmid per well is used in Tube A, the Lipofectamine 2000 to be used in Tube B will be 6.3 μL per well).

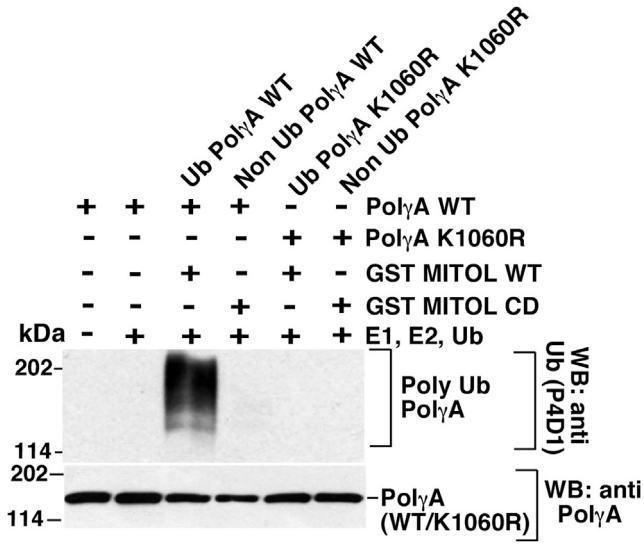

Figure 1. MITOL ubiquitylates PolγA *in vitro*

In vitro ubiquitylation was carried out using *in vitro* transcribed and translated PolγA (WT and KK1060R) as the substrate and recombinant wildtype (WT) or catalytically dead (CD) MITOL. (Top panel) Post-reaction, The ubiquitylated products were detected by carrying out Western blot analysis with anti-Ub (P4D1) antibody. (Bottom panel) Equal amounts of substrates used in each condition was determined by carrying out westerns with anti-PolγA antibody. This figure is reproduced from (Hussain et al., 2021) where it was presented as Figure S3A.

g. Transfer Tube B solution to Tube A (hence total volume total 500 μL) and mix well.
h. Incubate the reaction at room temperature inside the hood for 25 min. Meanwhile, aspirate complete media from 6 wells in the cluster plate and add 1.5 mL Opti-MEM to the wells.
i. Add plasmids-Lipofectamine 2000 complex to the cells (total 500 μL/well). Gently swirl and return the plate to the CO_2 incubator.
j. After 6 h remove the Opti-MEM from the wells and replace it with complete media (2 mL/well) and put back the cells in the incubator.
k. Prepare the lysate from the cells 24 h post-transfection.

Note: Steps mentioned here are for transfection of three different plasmids in a single well. Make sure to add proper controls for this experiment like His-Ubiquitin alone, His-Ubiquitin with E3 ligase without substrate and all three together. If the protocol is being adapted for other substrates or other E3 ligases, the amounts of plasmids to be transfected, the ratio of the plasmid to Lipofectamine 2000 and the time of incubation for the expression of proteins will have to be standardized. Certain E3 ligases ubiquitylate substrate protein and target the substrate for degradation. Hence, it is important to check different time points after transfection. Alternatively, the cells should be grown in presence of 10 μM of MG132 (a proteasome inhibitor) for the final 5 h of cell growth before lysates were prepared. This treatment will inhibit proteasomal degradation of the target protein.

4. Lysate preparation.
   a. Aspirate the media and wash the cells twice with 2 mL of ice-cold 1×PBS.
   b. Add 200 μL/well of M2 lysis buffer supplemented with 1× protease inhibitor cocktail (from 100× stock as recommended by the manufacturer) and PMSF (1 mM) to the cells.
   c. Scrape and transfer the cells from each well to one tube.
   d. Vortex at high speed for 30 s.
   e. Incubate on ice for 10 min.
   f. Centrifuge the sample at 17,000 × *g*, 4°C for 10 min.

g. Transfer the supernatant to a fresh prechilled tube and label it as cell lysate.

Note: Aliquoting is the preferred option for the storage of the supernatant. Ideally 50 μL aliquots should be made. A part of the supernatant (around 5–10 μL can be kept aside for protein estimation at this stage). Cell lysates can be stored for up to one month at −80°C. Avoid multiple freeze-thaw cycles. Not more than three freeze thaw cycles should be done.

h. Check the protein concentration using Bradford or BCA protein estimation method.
i. Confirm expression of the proteins by Western blot before proceeding to immunoprecipitation.

5. Western blot to check for PolγA expression in the lysates.
   a. Cells of each well were processed separately and equal amounts (typically 40–60 μg) of cell lysates were run in the 10% SDS-PAGE gel.
   b. The proteins were transferred onto the nitrocellulose membrane using Bio-Rad transfer apparatus using an overnight wet transfer protocol (at 40 mA per gel for 16 h).
   c. The membrane was blocked using 5% skim milk in 1× TBS + 0.1% Tween 20 and incubated in anti-Flag (1:1,000 dilution) or anti-Myc antibody (1:1,000 dilution), overnight with gentle rocking at 4°C. Substrate specific, anti-PolγA antibody can also be used to detect the levels of overexpressed PolγA.
   d. Next day, the blots were incubated in respective secondary antibodies (1:20,000 dilution) for an hour with gentle rocking at room temperature.
   e. The blots were washed and developed using an immobilon western chemiluminescent HRP substrate (from Millipore). Substitutes of the chemiluminescent HRP substrate are also available with Merck and Cell Signaling Technologies.
6. Immunoprecipitation followed by Western blot to detect ubiquitylated PolγA.
   a. For each reaction, equilibrate 5 μL of anti-Flag M2 affinity gel along with 10 μL of Sepharose CL6B (acting as inert support beads) in 500 μL M2 lysis buffer for 5 min.

   Note: Sepharose CL6B beads are recommended to be used along with the anti-Flag M2 affinity beads. Sepharose CL6B beads are used to visualize the Flag beads since anti-Flag affinity beads are translucent and it is hard to visualize the pellet during the aspiration step later in the protocol.

   b. Add the equilibrated anti-Flag M2 affinity beads and Sepharose CL6B into 100 μg–300 μg of cell lysates (the amount of cell lysate will depend on the level of expression of the PolγA as seen in via Western blot). This must be standardized if other substrates are used. Make up the volume to 500 μL using M2 lysis buffer.
   c. Incubate the sample at 4°C on an end-to-end rotor for 2 h.
   d. After incubation, wash the beads three times at 226 × *g*, 4°C with 1 mL of chilled M2 lysis buffer.
   e. Remove the supernatant completely without disturbing the beads. Add 10–20 μL of 2× SDS loading dye to the sample. Boil in boiling water for 10 min (a heating block can also be used).

   Note: The buffer removed in this step can be stored. In case the immunoprecipitation is not optimal – then the supernatant should be check for the unbound protein. In case the Flag tagged PolγA, is not immunopurified optimally, the pH of the M2 lysis buffer should be re-checked and/or the amount of anti-Flag M2 affinity gel increased to 7.5 μL or 10 μL per reaction.

   f. Separate immunoprecipitated samples along with a pre-stained protein marker on 10% SDS PAGE gel (SDS-PAGE gel was electrophoresed at 30 mA and gel was run out till 18 kDa protein to allow the visualization of the high molecular weight PolγA and its ubiquitylated forms). The proteins in the gels were transferred to a 0.45 μM nitrocellulose membrane (Bio-Rad).
   g. For Western blot, block the membrane using 3% skim milk powder in 1×TBS+ 0.1% Tween 20 for approximately one hour at room temperature with gentle shaking.

h. Use anti-Flag antibody (1:2,000 in 1×TBS + 0.1%Tween 20) antibody to detect the polyubiquitylation of PolγA. Alternately anti-PolγA or anti-Ubiquitin antibodies (both at 1:1,000 in 1×TBS+0.1%Tween20) could have been used to detect the polyubiquitylation of PolγA. Incubate the membranes with either of the antibodies at 4°C with gentle shaking overnight.
i. Wash the membranes three times with (1×TBS+0.1%Tween 20).
j. Incubate washed membranes with HRP-conjugated secondary antibodies (1:20,000 in 1×TBS+0.1%Tween 20) (details in key resources table) for 1 h at room temperature with gentle rocking.
k. Wash the blots three times and develop using an immobilon western chemiluminescent HRP substrate (details in key resources table).
l. After developing all the blots, the membrane was washed for 2 h with 1×TBS+0.1%Tween20 (every wash 15 min duration). Alternately the blot can be stripped with a commercially available stripping buffer (detailed in the key resources table) for 15 min at 37°C. In either case the blot was incubated only with the HRP-conjugated secondary antibody and the blot was developed to detect the antibody light chain. The light chain was probed to determine whether equal amount of antibody was used for each immunoprecipitation reaction.

Note: In cellulo ubiquitylation of FLAG-tagged PolγA is detected by using anti-Flag antibody (Figure 2). For PolγA we have also detected its ubiquitylated form using a substrate specific PolγA antibodies (detailed in Recourse table). However, not all the target-specific antibodies can detect the ubiquitylated forms. Hence it is important to try multiple antibodies against the substrate being tested. Alternatively, anti-ubiquitin-specific antibodies can be used. While P4D1 has been successfully tested for PolγA, other anti-ubiquitin which may be recommended are FK1 and FK2.

EXPECTED OUTCOMES

The poly-ubiquitylation of PolγA using *in vitro* method can be detected as a smear above the expected molecular weight of the target protein using anti-ubiquitin (Figure 1). For in cellulo ubiquitylation assays we have used anti-Flag antibody (as PolγA being expressed in cells is Flag tagged). A smear above PolγA indicating poly-ubiquitylation of the substrate *in cellulo* (Figure 2).

LIMITATIONS

The provided protocol is based on transient overexpression of multiple plasmids in HEK292T cells. Although the transient transfection system is widely used to study protein-protein interaction and other cellular functions, it is not considered to be an exact physiological condition. Overexpression of proteins using excess lipofectamine may increase cytotoxicity in some cell types, which might affect the results. Hence it is important to optimize the plasmid amount used for transfection.

In many cases, ubiquitylation leads to the degradation of the target protein. Hence the time point at which lysates are to be made needs to be standardized for individual proteins.

In some instances it is advisable to use chemical inhibitors like MG132 in assays to prevent degradation of the target protein and favor the accumulation of ubiquitylated target proteins.

Overexpression of Ubiquitin in cells might non-specifically add ubiquitin moieties to target proteins. It is important to use as less ubiquitin as possible and also include proper controls for reliable results. It is important to validate any ubiquitylation of substrates with both *in cellulo* ubiquitylation and *in vitro* ubiquitylation assays.

It is to be noted that the exact same protocol may not be applicable for every other E3 ligase and substrate. However similar protocols with modifications have been used by us to show how multiple

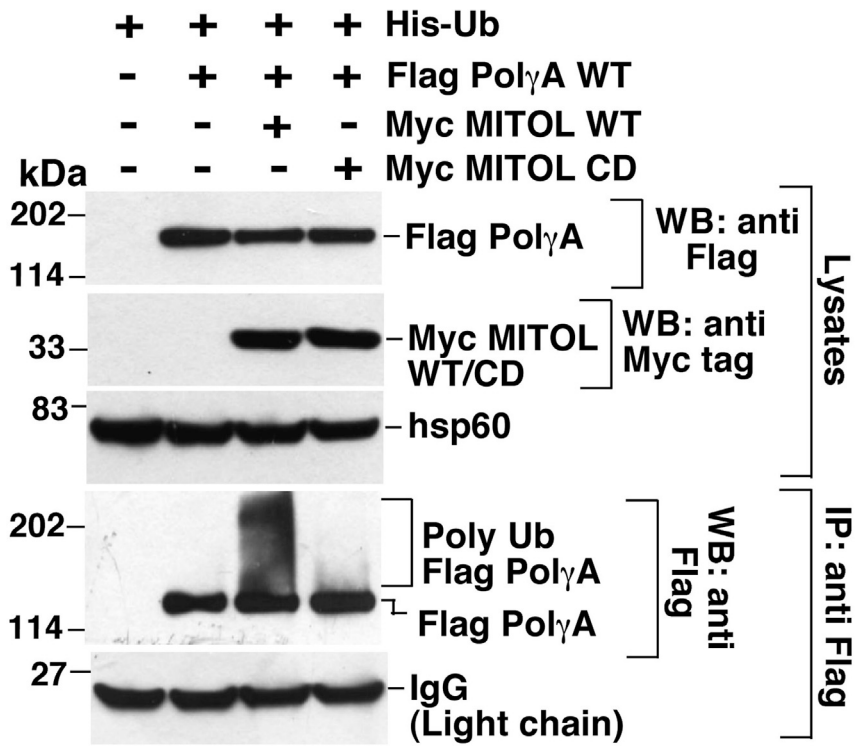

Figure 2. MITOL ubiquitylates PolγA *in cellulo*

(Top) Whole cell extracts were made from HEK293T cells transfected with His-tagged Ubiquitin (His-Ub), wildtype (WT) or catalytically dead (CD) Myc-tagged PolγA (Flag PolγA). Using the extracts western blots were carried out with antibodies against Flag, Myc tag and hsp60. Expression of hsp60 was monitored for equal protein loading control. (Bottom) Immunoprecipitations were carried out using anti-Flag beads. The immunoprecipitates were probed with antibody against Flag. Both ubiquitylated Flag-PolγA and the non-ubiquitylated Flag-PolγA have been indicated. The light chain was probed to show that equal amount of antibody was used for each immunoprecipitation reaction. This figure is reproduced from (Hussain et al., 2021) where it was presented as Figure 2B.

proteins have undergone ubiquitylation – like BLM (Kharat et al., 2016; Tikoo et al., 2013), p53 (Tripathi et al., 2019), c-Myc (Chandra et al., 2013) and c-Jun (Priyadarshini et al., 2018).

TROUBLESHOOTING

Problem 1

Non-specific ubiquitylation while using the *in vitro* method.

Potential solution

This can happen if too much recombinant ubiquitin is used in the reaction. It is essential to use a gradient of ubiquitin concentrations to get the optimal result. A negative control or non-target of the specific E3 ligase can be included to understand the specificity of the reaction. Sometimes E2 or E3 enzymes can non-specifically attach ubiquitin moieties to target proteins. It is important to make sure to use the optimal amounts of E2/E3 enzymes in the reaction. The ratio of E1:E2:E3 for individual E3 ligase/substrate combination has to be standardized by trail and error for different substrates.

May have to change how the recombinant substrate is purified. Usually substrates are purified from *E. coli* or yeast. It may be necessary to change to *in vitro* transcription and translation (TNT) system. Advantage of using *in vitro* TNT system is that it mimics eukaryotic transcription and translation, allowing post-translational modifications on target protein. If the ubiquitylation of target protein is

dependent on post-translational modification(s), then it's better to use target proteins generated in the TNT system.

Problem 2

No ubiquitylation or weak ubiquitylation.

Potential solution

Make sure to prepare active E3 ligase and store them in good conditions (such as multiple aliquots, each of 5 μL–10 μL, stored in −80° temperature to avoid multiple freeze thaw cycles that can affect proteins stability and activity). If possible, a known substrate can be included as a positive control to check E3 ligase activity.

Prepare the ubiquitylation assay buffer fresh every time.

It may also happen that the TNT reaction is giving non-specific or degraded substrate. In such case purification of the substrate has to be carried out from *E.coli* or yeast.

Further some E3 ligases only recognize phosphorylated substrates. In such cases the kinase needs to be identified. During *in cellulo* ubiquitylation, overexpression of the kinase may be required while carrying out the ubiquitylation assay.

Problem 3

Less cell lysate yield or increased cell death after transfection.

Potential solution

Never let HEK293T cells to reach 100% confluency. This can lead to induction of cellular stress followed by apoptosis. Always split them around 70% confluency.

Do not transfect cells which are more than 70% confluent. If needed, change media 4 h after transfection to reduce lipofectamine mediated cytotoxicity.

Consider making lysates at different time points post-transfection and checking the expression of the transfected substrate and ubiquitin.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sagar Sengupta (sagar@nii.ac.in).

Materials availability

Can be requested from Sagar Sengupta (sagar@nii.ac.in). While lab generated materials will be made available, commercial reagents which can be procured from vendors will not be distributed.

Data and code availability

The protocol described confirms that all data are available with the lead contact.

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AUTHOR CONTRIBUTIONS

M.H., Shabnam Saifi, and A.M. carried out cell biology and biochemical experiments. Sagar Sengupta designed the overall project and the experiments. M.H., Shabnam Saifi, A.M., and Sagar Sengupta analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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