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CoxII degradation assay to assess mitophagy

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ABSTRACT

This protocol details the procedure to assess mitophagy by analysing COXII degradation via Western blotting.

ATTACHMENTS

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MATERIALS TEXT

Buffers and reagents:

Growth media:

DMEM

A	B
FBS	10%
Glucose	4.5 g/l
GlutaMAXTM	1x
MEM NEAA	1x
HEPES	25 mM

45% D-( )-

- Glucose **Sigma Catalog #G8769**

GlutaMAX™ Supplement **Thermo Fisher**

- **Scientific Catalog #35050061**
-

MEAA (MEM Non-Essential Amino Acids) **Gibco - Thermo Fisher Catalog #11140050**

Antimycin A from Streptomyces sp. **Sigma –**

- **Aldrich Catalog #A8674** (made up in 100% Ethanol to 20 mg/ml), Oligomycin (Calbiochem, 495455; made up in DMSO to 10

Q-VD-

mg/ml) and OPh **MedChemExpress Catalog #HY-12305** (made up in DMSO to 10 mM).

- **Lysis buffer:** 1x LDS + 0.1 M DTT (diluted from 4x LDS (NP007; ThermoFisher); can be aliquoted and stored at -20 or -80 oC)
- 4-12% Bis-Tris NuPAGE gels (ThermoFisher)
- NuPAGETM Antioxidant (NP0005, ThermoFisher; use 0.5 ml/ 200 ml of gel running buffer)
- 20x NuPAGETM MOPS SDS running buffer (NP001, ThermoFisher)
- 20x NuPAGE transfer buffer (NP00061, ThermoFisher)
- PVDF destain: 40% Methanol, 7% Acetic Acid.
- 1x PBS
- 1x PBS/0.1% Tween20 (PBS/Tween)
- **Blocking buffer:** 5% skim milk in PBS/Tween (make fresh)
- ACTIN (Cell Signaling, 4967S), COXII (Abcam, ab110258), Parkin (Santa Cruz, sc-32282)
- Amersham ECL Prime Western Blotting Detection Reagent (RPN2232)

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Procedures: 4h 52m 10s

1 Seed the hela cells the day before the treatment day in 6 well plates.

1.1 Each well contain **2 mL** of growth media.

1.2 Seed 350,000 cells for penta KO expressing BFP-Parkin and GFP-OPTN or -NDP52.

1.3 Adjust the number of cells of other cell lines. So that the next day they are all in similar confluency with penta KO expressing BFP-Parkin and GFP-OPTN or -NDP52.

2 The next day, make sure the seeded cells are spreading out (not concentrated in the middle of the well because this can affect the results).

3 Aspirate off the old media and treat each well with **2 mL** of growth media containing **4 micromolar (µM)** Antimycin A, **10 micromolar (µM)** Oligomycin and **10 micromolar (µM)** QVD for desired period.

> **Note:** Make sure all drugs are vortexed well, mix the media well after adding each drug.

4 After the treatment, harvest the cells on ice by scraping.

4.1 Pre-chill eppies and 1x PBS on ice.

> **Note:** I normally put all the plates that need harvesting into a fridge and harvest one by one on ice.

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4.2 Aspirate the media thoroughly from the wells.

4.3 

Wash the wells with **1 mL** of cold 1x PBS.

> **Note:** Make sure swirl around after adding the PBS to wash the cells properly.

4.4 

Aspirate off the PBS and add another **1 mL** of cold 1x PBS.

4.5 Use a plastic cell scraper to scrape all the cells off the wells.

> **Note:** I use one scraper for each well. You can wash and reuse them again.

4.6 Transfer the cells-containing PBS to eppies.

4.7 

2m

Centrifuge the eppies at **3000 rpm** for **00:02:00** at **4 °C** .

4.8 Aspirate off PBS.

4.9 

10s

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Quickly centrifuge for **00:00:10** to spin down the residual PBS.

4.10 Aspirate off all the PBS.

5 Lyse the cell pellets in lysis buffer and boil the samples at **99 °C** with shaking for **00:07:00** . 7m

> **Note:** I use the plastic clips to make sure that the lids won't pop during heating.

6 Let the samples cool down and spin at max speed (**Room temperature**) for **00:01:00** . 1m

7 Estimate the protein concentration by nanodrop

> **Note:** Make sure the concentrations do not exceed **6 mg/mL** . If they do, dilute with lysis buffer and reheat them for a couple of minutes at **99 °C** with shaking.

8 

Aliquot **25 µg** of each sample into a new eppie and add 1x LDS to make up to **15 µL** .

9 Set up the gel tank with MOPs buffer.

> The inside chamber should be filled with 1x MOPs supplemented with antioxidants. The outside chamber doesn't need antioxidants.

10 

Wash each well with a glass syringe.

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11 Load markers and samples into the wells and run at 100V for **00:10:00** and 190V for **00:55:00** . 1h 5m

12 Subject the gels to wet transfer onto PVDF membrane using cold NuPAGE transfer buffer containing 20% Methanol for **01:00:00** at **Room temperature** . 1h

13  2m

After transfer, incubate the PVDF membrane with PVDF destain buffer on a shaker at **Room temperature** for **00:02:00** .

14 

Wash three times with PBS/Tween (5 min each wash).

14.1  5m

Wash the PVDF membrane with PBS/Tween for **00:05:00** (1/3).

14.2  5m

Wash the PVDF membrane with PBS/Tween for **00:05:00** (2/3).

14.3  5m

Wash the PVDF membrane with PBS/Tween for **00:05:00** (3/3).

15 Block with blocking buffer for **00:15:00** . 15m

16 Remove blocking buffer.

17 

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Rinse twice with PBS/Tween and wash twice with PBS/Tween and once with 1x PBS (5 min for each wash).

17.1 5m

Rinse the blocking buffer with PBS/Tween and wash with PBS/Tween for **00:05:00** (1/2).

17.2 5m

Rinse the blocking buffer with PBS/Tween and wash with PBS/Tween for **00:05:00** (2/2).

17.3 5m

Rinse the blocking buffer with 1x PBS and wash with 1x PBS for **00:05:00** .

18 Cut the PVDF membrane and put appropriate parts into different antibodies.

Note: In this case, it's ACTIN (1/5000), COXII (1/1000) and Parkin (1/1000) antibodies made up in 3% BSA in PBS/Tween.

19

Incubate on a **4 °C** shaker **Overnight** .

Note: To make sure we don't lose antibodies, I wet the tubs with PBS/Tween before putting in the antibodies.

20

The next day, recycle the antibodies back to their tubes.

21

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Wash the blots three times with PBS/Tween (5 min for each wash).

21.1 5m

Wash the blot with PBS/Tween for **00:05:00** (1/3).

21.2 5m

Wash the blot with PBS/Tween for **00:05:00** (2/3).

21.3 5m

Wash the blot with PBS/Tween for **00:05:00** (3/3).

22 1h

Incubate with appropriate HRP-conjugated secondary antibodies made up in blocking buffer for **01:00:00** .

23

Wash the blots twice with PBS/Tween, once with 1x PBS (5 min for each wash).

23.1 5m

Wash the blot with PBS/Tween for **00:05:00** (1/2).

23.2 5m

Wash the blot with PBS/Tween for **00:05:00** (2/2).

23.3 5m

Wash the blot with 1x PBS for **00:05:00** .

24 Develop the blots with ECL prime.

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