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CoxII degradation assay to assess mitophagy

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We use this protocol and it's working

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Abstract

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

Attachments

DOCX

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18KB

Materials

Buffers and reagents:

Growth media:

DMEM

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

- 45% D-( )-Glucose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G8769
- GlutaMAX™ Supplement **Thermo Fisher Scientific Catalog #**35050061
- MEAA (MEM Non-Essential Amino Acids) **Gibco - Thermo Fisher Scientific Catalog #**11140050
- Antimycin A from Streptomyces sp. **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A8674 (made up in 100% Ethanol to 20 mg/ml), Oligomycin (Calbiochem, 495455; made up in DMSO to 10 mg/ml) and Q-VD-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)

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 [M] 4 micromolar (µM) Antimycin A, [M] 10 micromolar (µM) Oligomycin and [M] 10 micromolar (µM) QVD for desired period.

Note

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

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

4.2 Aspirate the media thoroughly from the wells.

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

Note

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

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 Centrifuge the eppies at 3000 rpm for 00:02:00 at 4 °C . 2m

4.8 Aspirate off PBS.

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

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

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

Note: Make sure the concentrations do not exceed [M] 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.

Note

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.

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 After transfer, incubate the PVDF membrane with PVDF destain buffer on a shaker at Room temperature for 00:02:00 . 2m

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

14.1 Wash the PVDF membrane with PBS/Tween for 00:05:00 (1/3). 5m

14.2 Wash the PVDF membrane with PBS/Tween for 00:05:00 (2/3). 5m

14.3 Wash the PVDF membrane with PBS/Tween for 00:05:00 (3/3). 5m

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

16 Remove blocking buffer.

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

17.1 Rinse the blocking buffer with PBS/Tween and wash with PBS/Tween for 00:05:00 (1/2). 5m

17.2 Rinse the blocking buffer with PBS/Tween and wash with PBS/Tween for 00:05:00 (2/2). 5m

17.3 Rinse the blocking buffer with 1x PBS and wash with 1x PBS for 00:05:00 . 5m

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

Note

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

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

21.1 Wash the blot with PBS/Tween for 00:05:00 (1/3). 5m

21.2 Wash the blot with PBS/Tween for 00:05:00 (2/3). 5m

21.3 Wash the blot with PBS/Tween for 00:05:00 (3/3). 5m

22 Incubate with appropriate HRP-conjugated secondary antibodies made up in blocking buffer for 01:00:00 . 1h

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

23.1 Wash the blot with PBS/Tween for 00:05:00 (1/2). 5m

23.2 Wash the blot with PBS/Tween for 00:05:00 (2/2). 5m

23.3 Wash the blot with 1x PBS for 00:05:00 . 5m

24 Develop the blots with ECL prime.

25