Halo assay to assess mitophagy

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nguyen.tha[1]

[1]Laboratory of Michael Lazarou, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

COMMENTS 0

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WORKS FOR ME 2

ASAP workspace

nguyen.tha

ABSTRACT

This protocol describes how to assess mitophagy using Halo assay developed by Mizushima lab (DOI: 10.7554/eLife.78923).

ATTACHMENTS

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MATERIALS TEXT

Buffers and reagents:

Growth media:

A	B
DMEM with 10% FBS	
Glucose (Sigma, G8769)	4.5 g/l
1x GlutaMAXTM (ThermoFisher, 350500	
1x MEM NEAA (ThermoFisher, 11140-05	
HEPES (1688449)	25 mM

- Antimycin-A (Sigma, A8674; made up in 100% Ethanol to 20 mg/ml),
- Oligomycin (Calbiochem, 495455; made up in DMSO to 10 mg/ml)
- qVD (MedChemExpress, HY-12305; made up in DMSO to 10 mM)
- TMR-conjugated Halo ligand (Promega, G8251)
- *Lysis buffer*: (diluted from 4x LDS (NP007; ThermoFisher)

A
1x LDS
0.1 M DTT

can be aliquoted and stored at -20 or -80°C.

- 4-12% Bis-Tris NuPAGE gels (ThermoFisher)
- NuPAGE^{TM} Antioxidant (NP0005, ThermoFisher; use 0.5 ml/ 200 ml of gel running buffer)
- 20x NuPAGE^{TM} MOPS SDS running buffer (NP001, ThermoFisher)
- 20x NuPAGE transfer buffer (NP00061, ThermoFisher)

PVDF destain:

A
40% Methanol
7% Acetic Acid

- 1x PBS
- 1x PBS/0.1% Tween20 (PBS/Tween)
- *Blocking buffer*: 5% skim milk in PBS/Tween (make fresh)
- VCP (Cell Signaling, 2649), HALO (Promega, G9211)

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45% D-( )-Glucose **Sigma Catalog #G8769**
GlutaMAX™ Supplement **Thermo Fisher Catalog #35050061**
MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Scientific Catalog #11140050**
HEPES Buffer 1M Solution Cell Culture Grade MP Biomedicals **Fisher Scientific Catalog #ICN1688449**
Antimycin A from Streptomyces sp. **Sigma – Aldrich Catalog #A8674**
Oligomycin **Millipore Sigma Catalog #495455**
Q-VD-OPh **MedChemExpress Catalog #HY-12305**
HaloTag(R) TMR Ligand, 30ul **Promega Catalog #G8251**
NuPAGE™ LDS Sample Buffer **Thermo Fisher Scientific Catalog #NP0007**
NuPAGE Antioxidant **Thermo Fisher Scientific Catalog #NP0005**
NuPAGE™ MOPS SDS Running Buffer (20X) **Thermo Fisher Scientific Catalog #NP0001**
Tris-Glycine Transfer Buffer **Invitrogen - Thermo Fisher Catalog #NP00061**
VCP (7F3) Rabbit mAb **Cell Signaling Technology Catalog #2649**
Anti-HaloTag(R) Monoclonal Antibody **Promega Catalog #G9211**

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4h 57m 10s

Procedures

1 Generating cells expressing mitochondrially targeted Halo-GFP using pSu9-Halo-mGFP from Mizushima lab (Addgene #184905; DOI: 10.7554/eLife.78923).

2 Seed HeLa cells the day before the treatment day in 6 well plates.

- Each well contained 2 mL of growth media;
- Seed 350,000 cells for penta KO expressing BFP-Parkin and GFP-OPTN or -NDP52 and 380,000 cells for other knockout lines such as ATG13 KO/penta KO expressing GFP-NDP52;
- 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.

Note

3 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).

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4 Aspirate off the old media and treat each well with 1 mL growth media containing [M] 50 nanomolar (nM) TMR-conjugated Halo ligand.

Note

20m

5 Incubate in a normal TC incubator for 00:20:00.

6 Aspirate off the media and wash thoroughly.

6.1 Aspirate off the media and wash thoroughly with 1x PBS. (1/2)

6.2 Aspirate off the media and wash thoroughly with 1x PBS. (2/2)

6.3 Harvest the non-mitophagy-induced samples immediately by scraping (see step 8).

7 For mitophagy-induced samples, 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

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8 After the treatment, harvest the cells On ice by scraping.

Note

8.1 Pre-chill eppies and 1x PBS On ice .

Note

8.2 Aspirate the media thoroughly from the wells, wash the wells with 1 mL of cold 1x PBS*, aspirate off the PBS and add 1 mL of cold 1x PBS.

Note

8.3 After that, use a plastic cell scraper to scrape all the cells off the wells (I use one scraper for each well. You can wash and reuse them again). Transfer the cells-containing PBS to eppies.

2m

8.4 Centrifuge the eppies at 3000 x g, 4°C, 00:02:00 . Aspirate off PBS.

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10s

8.5 Quickly centrifuge for 00:00:10 to spin down the residual PBS. Aspirate off all the PBS.

7m

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

Note

1m

10 Let the samples cool down and spin at max speed (Room temperature) for 00:01:00 .

11 Estimate the protein concentration by nanodrop.

Note

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

13 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.
- Wash each well with a glass syringe.

1h 5m

14 Load markers and samples into the wells and run at 100V for 00:10:00 and 190V for 00:55:00 .

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15 Gels were then subjected to wet transfer onto PVDF membrane using cold NuPAGE transfer buffer containing 20% Methanol for 01:00:00 at Room temperature. 1h

16 After transfer,

16.1 Incubate PVDF membrane with PVDF destain buffer on a shaker at Room temperature for 00:02:00. 2m

16.2 wash with PBS/Tween: 15m
wash with PBS/Tween for 00:05:00. (1/3)
wash with PBS/Tween for 00:05:00. (2/3)
wash with PBS/Tween for 00:05:00. (3/3)

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

17 Remove blocking buffer,

17.1 Rinse:
Rinse with PBS/Tween. (1/2)
Rinse with PBS/Tween. (2/2)

17.2 Wash: 10m
Wash with PBS/Tween for 00:05:00. (1/2)
Wash with PBS/Tween for 00:05:00. (2/2)

17.3 Wash with 1x PBS for 00:05:00. 5m

18 Cut the PVDF membrane and put appropriate parts into different antibodies (in this case, it's VCP (1/1000) and 5m

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HALO (1/1000) antibodies made up in 3% BSA in PBS/Tween) to incubate on a 4 °C shaker Overnight .

Note

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

15m

19.1 Wash:

- wash the blots with PBS/Tween for 00:05:00 . (1/3)
- wash the blots with PBS/Tween for 00:05:00 . (2/3)
- wash the blots with PBS/Tween for 00:05:00 . (3/3)

1h

19.2 Incubate with appropriate HRP-conjugated secondary antibodies (1/10000 for HALO and 1/5000 for VCP) made up in blocking buffer for 01:00:00 .

20 Wash the blots and develop.

10m

20.1 Wash the blots,
wash the blots with PBS/Tween for 00:05:00 . (1/2)
wash the blots with PBS/Tween for 00:05:00 . (2/2)

5m

20.2 Wash the blots with 1x PBS for 00:05:00 .

20.3 Develop the blots with ECL prime.

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