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Halo assay to assess mitophagy

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We use this protocol and it's working

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Abstract

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

Attachments

DOCX

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20KB

Materials

Buffers and reagents:

Growth media:

	A	B
	DMEM with 10% FBS	
	Glucose (Sigma, G8769)	4.5 g/l
	1x GlutaMAXTM (ThermoFisher, 35050061)	
	1x MEM NEAA (ThermoFisher, 11140-050)	
	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)

45% D-( )-Glucose **Merck MilliporeSigma (Sigma-Aldrich) 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. **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A8674

Oligomycin **Merck MilliporeSigma (Sigma-Aldrich) 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

Troubleshooting

Procedures

4h 57m 10s

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

- Remember to include a set of samples as your non-mitophagy-induced controls

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).

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

- Aliquot the ligand into small aliquots and avoid freeze-and-thaw cycles.
- The ligand is sensitive to light so put the leftover away immediately after use and keep the media in the dark if you need to do a time course treatment.

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

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

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

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

Note

If you scan for fluorescence signal, from this step on consider keeping the samples away from light as much as possible.

8.1 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 .

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

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

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.

8.4 Centrifuge the eppies at 3000 x g, 4°C, 00:02:00 . Aspirate off PBS. 2m

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

9 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.

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

11 Estimate the protein concentration by nanodrop.

Note

Make sure the concentrations do not exceed 6 µL . If they do, dilute with lysis buffer and reheat them for a couple of minutes at 99 °C with shaking.

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.

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

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 HALO (1/1000) antibodies made up in 3% BSA in PBS/Tween) to incubate on a 4 °C shaker Overnight . 5m

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

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

19.1 Wash: 15m

- 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)

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 . 1h

20 Wash the blots and develop.

20.1 Wash the blots, 10m
wash the blots with PBS/Tween for 00:05:00 . (1/2)
wash the blots with PBS/Tween for 00:05:00 . (2/2)

20.2 Wash the blots with 1x PBS for 00:05:00 . 5m

20.3 Develop the blots with ECL prime.