**Halo assay to assess mitophagy**

Thanh Ngoc Nguyen (Laboratory of Michael Lazarou, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia) (nguyen.tha@wehi.edu.au)

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**Buffers and reagents:**

* + Growth media: DMEM with 10% FBS, 4.5 g/l Glucose (Sigma, G8769), 1x GlutaMAXTM (ThermoFisher, 35050061), 1x MEM NEAA (ThermoFisher, 11140-050), 25 mM HEPES (1688449)
	+ Antimycin-A (Sigma, A8674; made up in 100% Ethanol to 20 mg/ml), Oligomycin (Calbiochem, 495455; made up in DMSO to 10 mg/ml) and qVD (MedChemExpress, HY-12305; made up in DMSO to 10 mM)
	+ TMR-conjugated Halo ligand (Promega, G8251)
	+ 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)
	+ VCP (Cell Signaling, 2649), HALO (Promega, G9211),

**Procedures:**

1. Generating cells expressing mitochondrially targeted Halo-GFP using pSu9-Halo-mGFP from Mizushima lab (Addgene #184905; DOI: [10.7554/eLife.78923](https://doi.org/10.7554/elife.78923)).
2. HeLa cells were seeded the day before the treatment day in 6 well plates (each well contained 2 ml of growth media; 350,000 cells were seeded 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; the number of cells of other cell lines were adjusted so that the next day they are all in similar confluency with penta KO expressing BFP-Parkin and GFP-OPTN or -NDP52).
* Remember to include a set of samples as your non-mitophagy-induced controls
1. 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).
2. Aspirate off the old media and treat each well with 1 ml growth media containing 50 nM TMR-conjugated Halo ligand (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).
3. Incubate in a normal TC incubator for 20 min.
4. Aspirate off the media and wash thoroughly twice with 1x PBS. The non-mitophagy-induced samples can be harvested immediately by scraping (see step 8).
5. For mitophagy-induced samples, treat each well with 2 ml of growth media containing 4 μM Antimycin A, 10 μM Oligomycin and 10 μM QVD for desired period (make sure all drugs are vortexed well, mix the media well after adding each drug).
6. After the treatment, harvest the cells on ice by scraping. (If you scan for fluorescence signal, from this step on consider keep the samples away from light as much as possible)
* Pre-chill eppies and 1x PBS on ice. I normally put all the plates that need harvesting into a fridge and harvest one by one on ice.
* Aspirate the media thoroughly from the wells, wash the wells with 1 ml of cold 1x PBS (make sure swirl around after adding the PBS to wash the cells properly), aspirate off the PBS and add another ml of cold 1x PBS.
* 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.
* Centrifuge the eppies at 3000x g for 2 min at 4 oC. Aspirate off PBS.
* Quickly centrifuge for 10 sec to spin down the residual PBS. Aspirate off all the PBS.
1. Lyse the cell pellets in lysis buffer and boil the samples at 99 oC with shaking for 7 min (I use the plastic clips to make sure that the lids won’t pop during heating).
2. Let the samples cool down and spin at max speed (RT) for 1 min.
3. Estimate the protein concentration by nanodrop (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 oC with shaking).
4. Aliquot 25 μg of each sample into a new eppie and add 1x LDS to make up to 15 μl.
5. 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.
6. Load markers and samples into the wells and run at 100V for 10 min and 190V for 55 min.
7. Gels were then subjected to wet transfer onto PVDF membrane using cold NuPAGE transfer buffer containing 20% Methanol for 1 h at RT.
8. After transfer, PVDF membrane was incubated with PVDF destain buffer on a shaker at RT for 2 min, washed three times with PBS/Tween (5 min each wash), and blocked with blocking buffer for 15 min.
9. Remove blocking buffer, rinse twice with PBS/Tween and wash twice with PBS/Tween and once with 1x PBS (5 min for each wash).
10. 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 oC shaker overnight. To make sure we don’t lose antibodies, I wet the tubs with PBS/Tween before putting in the antibodies.
11. The next day, recycle the antibodies back to their tubes, wash the blots three times with PBS/Tween (5 min for each wash), incubate with appropriate HRP-conjugated secondary antibodies (1/10000 for HALO and 1/5000 for VCP) made up in blocking buffer for 1 h.
12. Wash the blots twice with PBS/Tween, once with 1x PBS (5 min for each wash) and develop the blots with ECL prime.