

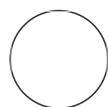


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Assessing autophagy using the HaloTag-LC3B cleavage assay

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ABSTRACT

Assay to detect HaloTag-LC3B during starvation autophagy based on
<https://doi.org/10.7554/eLife.78923>.

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Protocol status: Working
We use this protocol and it's working

Created: Oct 26, 2023

Assessing starvation autophagy using the HaloTag-LC3B 9h 52m

- 1 Generate HeLa cells expressing HaloTag-LC3B using pMRX-IP-HaloTag7-LC3 from Mizushima lab (Addgene #184899; DOI: 10.7554/eLife.78923).
- 2 Seed 400-450K cells in a six-well plate one day prior to the experiment.
- 3 Feed cells with  1 mL of complete DMEM for  01:00:00 1h
- 4 Replace with  1 mL of complete DMEM containing  50 nanomolar (nM) TMR-conjugated Halo ligand (Promega, GA1120) and incubate at  37 °C for  00:20:00 20m
- 5 Wash cells thrice with 1x PBS followed by incubation in  2 mL of EBSS (Gibco, 24010043) for  06:00:00 6h
- 6 Following starvation, harvest cells via cell scraping  On ice .
 - 6.1 Aspirate off DMEM and wash cells in  1 mL of ice cold 1x PBS.

- 6.2** Scrape cells from each well in  600 µL of ice-cold 1x PBS and transfer into chilled 1.5 ml Eppendorf tube.
- 6.3** To collect residual cells, add another  600 µL of ice-cold 1x PBS transfer into 1.5ml eppendorf tube.
- 6.4** Spin down cells at  10000 x g, 4°C for  00:01:00 and aspirate off PBS. 1m
- 6.5** Perform a quick-spin at  10000 x g, 4°C and aspirate off residual PBS.
- 6.6** Cell pellets can be frozen at  -20 °C or immediately used for immunoblotting
- 7** Lyse cells in 1 xLDS NuPAGETTM LDS Sample Buffer (Invitrogen, NP0007) containing  100 millimolar (mM) DTT and Boil at  99 °C on thermomixer with shaking.
- 8** Measure protein concentration using A280 setting on Nanodrop. Ensure sample concentration is between 4-6 mg/ml.
- 9** Load 20 ug of protein on 4-12% Bis-Tris gels (Invitrogen, WG1402A). Run gel using 3-step ramp up setting (100v for 10mins, 150v for 10mins and 180v for 55mins).
- 10** Transfer protein using wet transfer method onto an Immobilon-P PVDF Membrane (0.45 µm 1h

pore size) using a Criterion blotter (Bio-rad, 1704070), 100v for 01:00:00 .

- 11** Wash and block PVDF membrane: 25m
- 1x in PVDF destain for 00:01:00
 - 3x in TBS-T (1xTBS/0.05% Tween-20) for 00:02:00 each.
 - Block in 10% Milk/TBS-T for 00:20:00
 - 3x in TBS-T for 00:02:00 each
- 11.1** Cut the blots at the 65kDa molecular weight marker and incubate in primary antibodies made up in TBS-T/3% BSA overnight at 4 °C on rocking platform
- >65kDa section in VCP (Cell signalling, 2648S) (1/1000 dilution)
 - <65kDa section in anti-HaloTag (Promega, G9211) (1/100 dilution)
- 12** Following overnight incubation, recycle antibodies back into tubes and wash PVDF membrane. 2m
- 3x in TBS-T for 00:02:00 each
- 13** Incubate in anti-mouse HRP (Cell signalling, 7076S) and anti-rabbit HRP secondary antibodies (Cell Signalling, 7074S) for 01:00:00 1h
- HaloTag (1/10,000 dilution for secondary)
 - VCP (1/5,000 dilution for secondary)
- 14** Wash blots prior to developing in ECL prime (Cytiva, RPN2232SK) 4m
- 2x in TBS-T for 00:02:00 each.
 - 2x in TBS for 00:02:00 each.