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

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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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Assessing starvation autophagy using the HaloTag-LC3B clea... 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 [M] 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 NuPAGE™ LDS Sample Buffer (Invitrogen, NP0007) containing [M] 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.