Dec 01, 2025

HaloTag autophagy flux assay

Forked from HaloTag autophagy flux assay

DOI
https://dx.doi.org/10.17504/protocols.io.36wgq65xxlk5/v1

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Protocol Citation: Devin M. Fuller, Thomas Melia 2025. HaloTag autophagy flux assay. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq65xxlk5/v1

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

Created: April 03, 2025

Last Modified: December 01, 2025

Protocol Integer ID: 126118

Keywords: halotag autophagy flux

Funders Acknowledgements:
National Institutes of Health
Grant ID: R01 GM100930
National Institutes of Health
Grant ID: R35 GM153482
National Institutes of Health
Grant ID: DA018343
National Institutes of Health
Grant ID: F31 AG079606
National Institutes of Health
Grant ID: F31 DK136246
Aligning Science Across Parkinson's
Grant ID: ASAP-025173
Human Frontier Science Program
Grant ID: LT000056/2020-C

Abstract

Pule-chaseable method for quantifying autophagy flux

Materials

Cell culture materials:
DMEM (Thermo Fisher Scientific, 11965-092)
FBS (Thermo Fisher Scientific, 16140-071)
Penicillin/Streptomicin (10,000 U/mL; Thermo Fisher Scientific, 15140122)
PBS (Thermo Fisher Scientific, 10010023)
Earle's Balanced Salt Solution (EBSS; Thermo Fisher Scientific, 24010043)

Transfection Reagents:
Opti-Mem (Thermo Fisher Scientific, 31985062)
Lipofectamine 3000 (Thermo Fisher Scientific, L3000008)
Dharmafect (Horizon Discovery, T-2001-01)

Lysis Buffer:
lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40) with Protease Inhibitor Cocktail
Tris (American Bio, AB02000-05000)
NaCl (Sigma-Aldrich, S9888-25G)
EDTA (Sigma-Aldrich, 3690)
Nonidet P40 Substitute (Roche, 11754599001)
COmplete mini EDTA Free (Roche, 11836170001)

Other reagents:
TMR-conjugated HaloTag ligand (Promega, G8251)

Seeding plates

1 Seed 50,000 HEK293 cells in each chamber of a 6-well treated tissue culture plate. Maintain cells in DMEM + 10% fetal bovine serum (FBS) + 1x penicillin and streptomycin.

2 Grow for 2 days until cells are ~80% confluent

Transient Transfection

30m

3 If cells do not need to be transfected, skip to autophagy induction and cell collection

4 Wash cells into DMEM + 10% FBS without antibiotic prior to transfection (at 60-70% confluence).

5 Prepare reagents for transfection with either lipofectamine 3000 or dharmafect.

5.1 For transfected plasmids, each well requires 1 µg of target DNA construct. Prepare a master mix consisting of 1 µg of DNA, 125 µL of opti-mem low serum medium, and 2 µL of P3000 reagent per well. Prepare an additional mix consisting of 125 µL of opti-mem and 2 µL of lipofectamine 3000 per well. Combine the DNA + P3000 and lipofectamine solutions together, and allow to incubate for 00:10:00 at room temperature. 10m

5.2 For siRNA, transfect a final concentration of [M] 25 nanomolar (nM) of the indicated siRNA. Prepare a master mix consisting of siRNA and 125 µL of opti-mem low serum medium per well. Prepare an additional mix consisting of 125 µL of opti-mem and 4 µL of Dharmafect (Horizon Discovery) per well. Combine the DNA and dharmafect solutions together, and allow to incubate for 00:10:00 at Room temperature. 10m

6 After 00:10:00, dispense 250 µL of combined mixture into each well to be transfected. 10m

7 Return plates to incubator, and incubate for 1-2 days (minimum of 2 days for siRNA KD) before conducting experiment

Autophagy Induction and Cell Collection

4h 38m

8 Replace growth medium with labeling medium consisting of growth medium + 100 nM Janelia Fluor 549-conjugated HaloLigand.

9 Incubate at 37 C for 00:30:00 . 30m

10 Aspirate off labeling medium and wash 2x with PBS or growth medium.

11 Replace the media with Earle's Balanced Salt Solution (EBSS) to start the experiment.

12 Return cells to incubator for 04:00:00 . 4h

13 Supplement lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40) with Protease Inhibitor Cocktail (Roche) and chill On ice .

14 Collect cells via scraping into ice-cold PBS.

15 Centrifuge at 500 x g, 4°C, 00:03:00 , aspirate the excess PBS. 3m

16 Pipette 150 µL lysis buffer onto cell pellet, pipette mixture up and down 10 times with a P-200 pipette tip

17 Incubate Eppendorf tube On ice for 00:05:00 . 5m

18 Determine protein concentration in sample using Bradford Assay.

19 Prepare samples at desired concentration, add 1 mM DTT and 1x LDS loading buffer.

HaloTag gel shift measurement and quantification

5m

20 Incubate samples at 95 °C for 00:05:00. 5m

21 During this incubation, prepare gel apparatus with NuPAGE™ Bis-Tris Mini Protein Gels, 4–12%, 1.0–1.5 mm (ThermoFisher) and 1x MOPS running buffer.

22 Load samples into gel and run until dye front reaches bottom (180 V, roughly 60 min). Rinse in deionized water before imaging on a ChemiDoc using the AlexaFluor 548 channel. Use AutoExpose protocol unless trying to image particularly faint bands.

23 If immunoblotting with this gel, proceed to the transfer step.

24 Open image file in Fiji, define rectangular selection that encompasses the first lane, and use the Gel Analyzer tool to highlight each lane before plotting the trace.

25 In the trace window, define a baseline, and then measure the area under each section of the curve that describes the band of interest. The highest MW band should represent the full length HaloTag reporter, while the lowest MW band should represent processed HaloTag.

26 Calculate the fraction of total Halo signal constituted by the lowest band over the total halo signal to quantify autophagy flux.

Protocol references

https://doi.org/10.7554/eLife.78923

Acknowledgements

This work was supported by grants from the National Institutes of Health (R01 GM100930 and R35 GM153482 to TJM; R01 GM151829 to JB; DA018343 to PDC), F31 AG079606 to DMF and F31 DK136246 to JLK. This research was also funded in part through Aligning Science Across Parkinson's (ASAP-025173 to TJM and PDC) through the Michael J. Fox Foundation for Parkinson's Research (MJFF) and the Howard Hughes Medical Institute (HHMI; PDC). FS acknowledges support from the Human Frontier Science Program (LT000056/2020-C). JB acknowledges support by the Wellcome Leap Foundation. Imaging was supported by the Yale Center for Cellular and Molecular Imaging (both the fluorescence and electron microscopy facilities). We also thank the MS & Proteomics Resource at Yale University for providing the necessary mass spectrometers and the accompany biotechnology tools funded in part by the Yale School of Medicine and by the Office of The Director, National Institutes of Health (S10OD02365101A1, S10OD019967, and S10OD018034). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.