Dec 01, 2025

# APEX2-based proximity biotinylation of ATG2A

Forked from APEX2-based proximity biotinylation of NLRP3 and P4C (SidC) during inflammasome activation

DOI
dx.doi.org/10.17504/protocols.io.4r3l2686qv1y/v1

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**Protocol Citation:** Devin M. Fuller, Thomas Melia 2025. APEX2-based proximity biotinylation of ATG2A . **protocols.io** **https://dx.doi.org/10.17504/protocols.io.4r3l2686qv1y/v1**

**Manuscript citation:**
Fuller Devin M, Wu Yumei, Schueder Florian, Rasool Burha, Nag Shanta, Korfhage Justin L, Garcia-Milian Rolando, Melnyk Katerina D, Bewersdorf Joerg, De Camilli Pietro, Melia Thomas J (2025) ATG2A engages RAB1A and ARFGAP1 positive membranes during autophagosome biogenesis eLife 14:RP107316

https://doi.org/10.7554/eLife.107316.1



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

**Created:** March 31, 2025

**Last Modified:** December 01, 2025

**Protocol Integer ID:** 125791

**Keywords:** APEX2, proximity labeling, ATG2A, proximity biotinylation of atg2a, based proximity biotinylation, other target protein, proximity labeling for the discovery, protein, atg2a, based proximity labeling, apex2, apex2 protocol

**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

APEX2-based proximity labeling for the discovery of proteins proximal to ATG2A. Aspects of this APEX2 protocol can be applied to other target proteins.

## 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)

**Chemicals, Peptides, and Recombinant Proteins**

Protease Inhibitor Cocktail **Roche** **Catalog #**11873580001
tris(2-carboxyethyl)phosphine (TCEP) **Gold Biotechnology** **Catalog #**TCEP2
Sodium Chloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S9888
MOPS **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M1254

DTT (GoldBio, DTT10)
Biotinyl Tyramide (Sigma-Aldrich, SML2135)
Potassium chloride, KCl (Sigma #P9541)
Sodium chloride, NaCl (Sigma 105012)
Trolox (Cayman chemicals, 10011659)
Sodium azide (Sigma S2002)
(+)-Sodium L-ascorbate (Sigma A4034)
Sodium carbonate (7527-04)
Urea (Sigma U5378)
SDS, Sodium Dodecyl Sulfate (Bio-Rad #1610302)
BSA, 200 mg/mL standard (Sigma, P5369)

**Buffers and solutions**
Lysis buffer: 50 mM Tris HCl pH 7.5, 150 mM NaCl, 1.0% (v/v) SDS, 1.0 % (v/v) NP-40, 1X cOmplete™ Protease Inhibitor Cocktail
30% (wt/wt) $H_2O_2$ reagent (Sigma H1009; NOTE: Do not keep more than ~6 months)

**Other**
Streptavidin-coated agarose beads: Pierce (#88817)

## Troubleshooting

## Cell line construction and validation (brief overview)

1 APEX2-GFP-ATG2A was stably introduced into HEK293 ATG2 DKO cells by viral transduction.

An extended protocol for producing virus is available here: https://www.addgene.org/protocols/lentivirus-production/?utm_term=&utm_campaign=Primary+Ad+Group:+Website,+blog,+collections.&utm_source=adwords&utm_medium=ppc&hsa_acc=3245806047&hsa_cam=112133441&hsa_grp=63724608643&hsa_ad=320492093642&hsa_src=g&hsa_tgt=dsa-596073738403&hsa_kw=&hsa_mt=&hsa_net=adwords&hsa_ver=3&gad_source=1&gclid=Cj0KCQjwztOwBhD7ARIsAPDKnkBDRe1ZLOhpLmjku0S2lwpO2RobkQ5ylaJPMUZ4Sofxe5MhJOULGNAaAvVrEALw_wcB

1.1 This cell lines was cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 10% fetal bovine serum, and Penicillin-Streptomycin. They were maintained in a 5% $CO_2$ incubator at 37 $^{o}$C.

2 Check protein expression levels by comparing ATG2 DKO vs ATG2 DKO + APEX2-GFP-ATG2A cells by immunoblotting.

3 Check protein localization by confocal microscopy.

4 Validate functional reconstitution of APEX2-GFP-ATG2A in ATG2 DKO HEK293 cells by immunoblotting against LC3B and P62. The protein levels should return to WT levels.

5 ATG2 DKO cells stably expressing APEX2-EGFP-ATG2A were cultured for two weeks in media containing
heavy, medium, or light isotopes of arginine and lysine (Cambridge Isotope Laboratories, CLM-2265-H-PK, DLM-2640-PK, CNLM-291-H-PK; Sigma-Aldrich, 608033). The light isotope condition (normal DMEM) was used as a no labeling control (-$H_2O_2$), the medium isotope condition was used for a full labeling reaction in complete media, and the heavy isotope condition was used for a full labeling reaction in starvation conditions (EBSS).

## APEX2 proximity biotinylation

6 Prepare the following ahead of time: Biotin-phenol/biotin tyramide (500 mM in DMSO, aliquot, store at -80 °C)

7 Grow cells to fully confluent in 3×15-cm dishes per condition. Cells should be seeded in 20 mL complete DMEM.

8 Prepare RIPA
*lysis buffer: 50 mM Tris HCl pH 7.5, 150 mM NaCl, 0.1% (wt/v) SDS, 0.5% Sodium Deoxycholate (wt/v), 1% TritonX-100, 1X cOmplete™ Protease Inhibitor Cocktail

Note

Lysis buffer can be kept slightly chilled but cannot be put on ice, because the 1% SDS will precipitate. Work SDS powder in a hood, if applicable. Detergents and other components can be dissolved overnight, but the protease inhibitor cocktail should be added immediately before proceeding.

9 For the cell harvesting day, freshly prepare: 100X Trolox in DMSO (500 mM), 100X Sodium ascorbate in PBS (1 M), 100X sodium azide in PBS (1 M). Each of these solutions is 1:100X, so prepare enough for the amount of quenching buffer used (35 mL buffer per replicate).

10 Stagger treatments so that only 3-4 plates (for example, 1 condition) are harvested at a time.

10.1 Add 20 µL biotin phenol (BP, 500 mM) to cells 30 mins prior to collecting them. For the starvation condition, replace the media with EBSS for 1.5 hrs, then add 20 µL biotin phenol for the remaining 30 min.

Note

BP is not very soluble in cell culture medium and will likely crash out before dissolving into solution, so add it to the cells while swirling the plate and continue swirling until all it goes into solution.

11 Just before harvesting the cells, prepare the following:

11.1 100 mM $H_2O_2$ in DPBS (10 uL of 30% $H_2O_2$ into 990 µL DPBS). Prepare individual pipettes with 200 uL of $H_2O_2$ for each plate being harvested at a time.

11.2 3 falcon tubes per plate, with 10 mL ice-cold quenching buffer in each.

Note

Make up fresh batches of quenching buffer by diluting 100x Trolox, 100x Sodium ascorbate, and 100X sodium azide into ice-cold PBS with vigorous stirring (to 1X).

11.3 Trays of ice for harvesting cells.

12 Bring cells to the bench and harvest them.

12.1 Quickly pipette $H_2O_2$ into each of the plates, swirl, and incubate at RT for 1 min, rocking. Do not add $H_2O_2$ to the light isotope condition, which serves as a negative control.

12.2 Pour off the solutions into appropriate waste containers and quickly add ice-cold quencher solution to each plate, and place them on ice.

12.3 As starved cells are loosely adherent, do not perform all washes in the dish. Instead, scrape the cells in 10 mL of quencher buffer (5 mL initially, 5 mL for residual cells), collect in 15 mL falcon tubes, and centrifuge (100 xg, 4 $^{O}$C , 5 min).

12.4 Add 10 mL of quencher buffer, resuspend cells. Centrifuge cells (100 xg, 4 $^{O}$C , 5 min). (1/2)

12.5 Add 10 mL of quencher buffer, resuspend cells. Centrifuge cells (100 xg, 4 $^{O}$C , 5 min). (2/2)

12.6 Aspirate the quencher solution and resuspend the cells in 800 uL of RIPA lysis buffer. For each condition, combine the cell lysates from the three 15 cm plate into one tube.

12.7 Incubate the lysates for 10 min on ice. Pipette up and down to ensure homogenization.

12.8 Pipette the samples into 1.5 mL eppendorf tubes. Centrifuge lysates (16000 xg, 4 $^{O}$C , 10 min). Pipette clarified supernatants into new eppendorf tubes.

12.9 Flash-freeze pellets in liquid nitrogen. Store at -80 $^{O}$C until ready for immunoprecipitation.

13 Repeat with additional replicates at staggered timepoints to prevent an overwhelming amount of simultaneous bench work.

## Mass spec prep

2h 15m

14 Thaw cell lysates on ice, perform Bradford Assay to quantify protein concentrations.

15 Gently vortex the stock of magnetic streptavidin beads for ~30 s to thoroughly resuspend them. Aliquot 200 µL beads (slurry) into an eppendorf tube using a wide bore pipette tip. Wash 2x with lysis buffer.

16 Combine the heavy, medium, and light isotope conditions by adding an equal mass of protein (~4 mg) of each condition to the magnetic streptavidin beads. Save the remaining lysates to run separately as quality control. Incubate the beads overnight at 4 $^{o}$C, rotating.

17 Wash the beads thoroughly with several solutions, using a magnetic rack to pellet the beads. Resuspend the magnetic beads with a wide bore pipette when changing solutions and place them on an end-over-end rotator while resuspending all of the replicates. Ensure that the beads don't aggregate, and if they do, gently pipette up and down with a wide bore P1000 to disperse them. Perform the washes at 4 $^{o}$C.

17.1 Wash with 1 mL of RIPA lysis buffer (1/2)

17.2 Wash with 1 mL of RIPA lysis buffer (2/2)

17.3 Wash with 1 mL of 1 M KCl

17.4 Wash with 1 mL of 0.1 M Sodium Carbonate ($Na_2CO_3$)

17.5 Wash with 1 mL of 2 M Urea in 10 mM Tris (pH 8.0)

17.6 Wash with 1 mL of RIPA lysis buffer (1/2)

17.7 Wash with 1 mL of RIPA lysis buffer (2/2)

18 Elute the sample by incubating the beads in 2x LDS loading buffer supplemented with 2 mM Biotin and 20 mM DTT for 10 min at 95 $^{o}$C.

19 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.

20 Load the eluted sample into the gel and run until dye front is approximately 2 cm from the well (120 V, 10 min).

21 Stain the gel for 1 hr with Simply Blue Safestain.

22 Destain the gel in distilled water overnight. Excise the gel plug and place in an eppendorf tube. Send to the appropriate MS center for in gel digestion and analysis. Note that sample preparation may differ based on the facility used. Consult with the center's technician before starting this protocol.

## Protocol references

https://pubmed.ncbi.nlm.nih.gov/25419960/
https://pubmed.ncbi.nlm.nih.gov/26866790/

## 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.