**Detailed protocol for using anti-phospho-ATG16L1 antibody (ab195242) to detect autophagy induction**

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**Abstract**

Autophagy is a degradative program that maintains cellular homeostasis and is responsible for clearing potentially toxic elements from the cell. Defects in autophagy have been described in pathophysiology including neurodegeneration, cancer, and inflammatory bowel disease. However, analysis of autophagy rates can be challenging, particularly in rare cell populations or *in vivo*, due to inherent limitations of current tools available for measuring autophagy pathway induction. Here, we describe a novel method to monitor autophagy by measuring post-translational modification of the protein ATG16L1. We developed and characterized a monoclonal antibody that can detect phospho-ATG16L1 endogenously in mammalian cells by western blot, immunofluorescence, and immunohistochemistry. The detailed protocol for these assays utilizing the phospho-antibody is described in the text below.

**Introduction**

As described in our Nature Methods publication “An antibody for analysis of autophagy induction”, detection of phospho-ATG16L1S278 (pATG16L1) can be utilized as an alternative method to measure autophagy induction with unique advantages over commonly employed LC3-based assays. This protocol describes our step-by-step optimized protocols for the usage of the rabbit monoclonal phospho-antibody (Abcam, ab195242) in detecting pATG16L1 signal by western blot, immunofluorescent microscopy (IF), and immunohistochemistry (IHC) assays.

**Reagents and equipment**

Solutions and recipe

PBS (Phosphate buffered saline)

* 154mM NaCl, 1.06mM KH2PO4,2.96mM Na2HPO4. Adjust pH to 7.4

TBST (Tris buffered saline with Tween)

* 20mM Tris, 171mM NaCl, 0.05% Tween 20. Adjust pH to 7.6

Digitonin permeabilization buffer

* 50ug/mL digitonin in PBS
* Digitonin stock solution is 12.5mg/ml in ethanol

p16L-BB (pATG16L1 blocking buffer for IF)

* 0.1% BSA, 1x abcam blocking buffer in PBS

IFBB (IF blocking buffer)

* 2% FBS, 1% BSA in PBS

TbT (Tris buffered NaCl with Tween) – make fresh each time

* 50mM Tris, 300mM NaCl, 0.1% Tween 20. Adjust pH to 7.6

Laemmil Sample Buffer

* 200mM Tris-HCl pH 6.8, 300mM NaCl, 0.1% Tween 20. Adjust pH to 7.6

2.5% BSA (bovine serum albumin, Sigma, A3294) in TBST

2% skim milk in TBST

4% PFA (paraformaldehyde, Sigma-Aldrich, 158127)

10x Abcam blocking buffer (Abcam, ab126587)

Antibodies

pATG16L1 (Ser278) rabbit monoclonal antibody (Abcam, ab195242)

LC3B rabbit monoclonal antibody (MBL, PM036)

p62/SQSTM1 mouse monoclonal antibody (MBL, M162-3)

Other reagents

Bio-Rad Clarity MaxTM ECL substrate (1705062S)

ProLongTM Gold Antifade (P36930)

Optimal cutting temperature compound

D-sucrose (Fisher Scientific, BP200-212)

Tissue-culture treated plastic coverslips (ibidi, 10814)

Superfrost Plus microscope slides (Fisher Scientific, 12-550-15)

Machines and Equipment

Bio-Rad TransBlot® TurboTM

Western blot film (Diamed, DIAMED810)

Zeiss LSM 800 AxioObserverZ1 Confocal MicroscopeBio-Rad ChemiDocTM imaging system

Rocking platform (Boekel model 260350)

**Procedure**

**Western Blot**

* After treatment, wash cells twice with PBS then lyse cells directly in 1x Laemmil Sample Buffer (approx. 100uL for a single well on a 12-well plate). Washing twice is recommended since residual BSC from culture media can interfere with ATG16L1 detection on western blot
* Boil samples for 10 minutes at 95˚C
* Resolve samples on SDS-polyacrylamide gel, 6%-18% gradient or 10% if using single phase gel. Gel format: Biorad mini gel.
* Transfer proteins onto PVDF membrane with Bio-Rad TransBlot® TurboTM system. Transfer settings: 0.7A, 25V, 25 minutes for a single gel.
* Dilute 10x Abcam blocking solution (ab126587) with PBS and block the membrane in diluted blocking buffer for 30 minutes. **Use of ab126587 is strongly recommended as it significantly reduces background for pATG16L1 blots**.
* Wash the membrane 5x5 minutes with TBST
* Dilute pATG16L1 antibody at 1:2000 in 2.5% BSA in TBST solution.
* Incubate membrane overnight at 4˚C with pATG16L1 primary antibody solution on rocking platform.
* Wash the membrane 5x5 minutes with TBST
* Dilute goat anti-rabbit HRP-conjugated secondary antibody at 1:5000 to 1:10000 (exact dilution of the secondary antibody is dependent on factors such as sample concentration and signal detection method, should be optimized empirically by individual researchers) in 2.5% skim milk in TBST solution.
* Incubate membrane at room temperature for 45 minutes with secondary antibody solution.
* Wash the membrane 5x5 minutes with TBST
* Develop signal with strong ECL substrate (we recommend ClarityMaxTM ECL from Bio-Rad, 1705062S) then detect signal with digital western blot imager (Bio-Rad ChemiDocTM).

**Immunoflurescent Microscopy**

For an unknown reason we always had a difficult time detecting pATG16L1 in HEK293A cells, for IF analysis avoid this cell line if possible.

We have developed a specific blocking buffer (p16L-BB) that works well with the pATG16L1 antibody but not as good with other antibodies. When co-staining with another antibody we block with our regular IF blocking buffer (IFBB) and incubate with the other primary antibody first, followed by blocking with p16L1-BB and incubation with pATG16L1 primary antibody. Secondary antibody incubation was also done in p16L1-BB. The following protocol is taken from experiments where we co-stained pATG16L1 with a monoclonal mouse p62 antibody (MBL, M162-3).

* Seed cells on tissue-culture treated plastic coverslips (ibidi, Cat# 10814) 2 days prior experiment to achieve a confluence of ~75% on experiment day.
* After experiment, fix cells with 4% PFA solution for 15 minutes
* Remove majority (~90%) of the PFA solution via aspiration and add PBS to fully cover the coverslips. At this stage, the coverslips can be stored at 4˚C for up to 30 days.
* Quick wash coverslips once with PBS
* Permeabilize with digitonin solution (50ug/mL in PBS) for 10 minutes
* Quick wash coverslips once with PBS
* Incubate coverslips in IFBB (2% FBS, 1% BSA in PBS) for 30 minutes
* Incubate coverslips with mouse p62 antibody (MBL, M162-3. 1:500 diluted in IFBB) for 1 hour
* Quick wash coverslips 3 times with PBS
* Incubate coverslips in p16L1-BB (0.1% BSA, 1x abcam blocking buffer ab126587 in PBS) for 30 minutes
* Incubate coverslips with pATG16L1 antibody (1:150 diluted in p16L1-BB) for 1 hour
* Quick wash coverslips 3 times with PBS
* Incubate coverslips in secondary antibodies (Alexa Fluor 647 goat anti-rabbit, Alexa Fluor 488 goat anti-mouse, diluted 1:1000 in p16L1-BB) for 60 minutes.
* Quick wash coverslips 3 times with PBS
* Incubate coverslips with DAPI (0.1ug/mL) for 2 minutes
* Quick wash coverslips 3 times with PBS
* Mount onto glass microscope slides with ProLongTM Gold Antifade mounting reagent (P36930).

**Immunohistochemistry**

Tissue harvest and fixation

*Quadriceps muscles – frozen sections*

* Sacrifice mice by cervical dislocation and immediately harvest quadriceps muscles.
* Apply a layer of optimal cutting temperature compound (OCT) to fully cover the harvested muscle then snap freeze in liquid nitrogen-cooled isopentane bath
* Cut into 10um thick slices and mount onto glass slides
* Rinse once with PBS
* Dry slides for at least 20 minutes to remove all moisture
* Fix with 4% PFA for 25 minutes

*Quadriceps muscles – paraffin embedded sections*

* Sacrifice mice by cervical dislocation and immediately harvest quadriceps muscles.
* Fix in 10% formalin solution for at least 48 hours
* Incubate with 20% sucrose in PBS solution for 72 hours, change fresh solution every 24 hours (optional)
* Dehydrate and paraffin embed tissue samples
* Cut into 4um thick slices and mount onto glass slides
* Antigen-retrieval in pH 9.0 EDTA solution, at 110°C for 12 minutes in a microwave processor (Histo5, Milestone).

*Liver and brain*

* Deeply anesthetize the mice as per animal care protocol.
* Open the chest cavity and cut the diaphragm carefully taking extra effort to not damage the heart, lungs, or liver.
* Insert a 26G needle into the left ventricle of the heart horizontally.
* Cut the right atrium to open the circulatory system.
* Perfuse with 0.1M phosphate buffer saline (PBS) and 4% Paraformaldehyde (both at pH = 7.4) for 6 and 15 minutes respectively at a rate of 7ml/min. CRITICAL: Well-perfused animals should have white extremities and lungs, and lighter liver.
* Remove the brain using rongeurs while taking care to not damage the brain.
* Use a small spatula to remove the brain from the cranium. CRITICAL: Always apply pressure away from the brain and ensure the dura mater and meninges have been cut.
* Place the brain to postfix in 4% paraformaldehyde for 1 hour on a shaker at room temperature.
* Cryopreserve the brain in 10ml of 30% sucrose/0.1% sodium azide in 0.1M PBS in 15ml falcon tube at 4oC until sunken within the tube.

Sectioning – brain

* Place dry ice in the stage of a freezing microtome and wait until it is frosty.
* Place several drops of water on the frozen stage and immediately place and hold the brain on stage until immobilized.
* Drop more water on the brain until sufficient coverage around the base of the brain is reached
* Cover the brain with crushed dry ice to accelerate freezing. CRITICAL: Make sure there is always dry ice in the stage to avoid thawing and unwanted antigen damage.
* Remove dry ice from the brain but make sure to leave enough in the stage. A frozen brain should be white in colour.
* Set the microtome to cut at 30uM and cut the brain coronally all the way to the cerebellum at a sampling fraction of 12.
* Cut sections are to be immediately placed in 0.1M PBS with 0.1% sodium azide for storage at 4oC until immunostaining.

Antibody staining

* Rinse samples 3 times with TbT (*Quadriceps samples only*)
* Incubate in 3% H2O2 in PBS for 10 minutes (*Quadriceps samples only*)
* Wash samples 3 times with TbT
* Block with 5% BSA in PBS for 2 hours
* Incubate with primary antibody (diluted in 5% BSA in PBS) overnight at 4°C:
	+ For staining with pATG16L1: 1 in 150 (Abcam, Ab195242)
	+ For staining with LC3B: 1 in 1000 (MBL, PM036)
* Wash 3 times with TbT
* Incubate with secondary antibody (Alexa Fluor 647 or 555 goat anti-rabbit, diluted in blocking buffer) for 60 minutes.
* Wash once with TbT
* Stain with DAPI for 2 minutes
* Wash 5x5 minutes with TbT
* Mount plastic coverslip on top of tissue section with ProLongTM Gold Antifade mounting reagent

**Troubleshooting**

General

* The phospho-serine 278 residue this antibody detects is only present on the  isoform of ATG16L1. Hence in western blots only one band will be present for pATG16L1 as opposed to the usual 2 bands for total ATG16L1. This should be kept in mind when working with ATG16L1 knockouts/reconstituted cell lines or animals.
* For western blot and IF analysis, a simple reliable positive control for pATG16L1 detection is 1-3 hour amino acid starvation.

Western blot

* Diluted antibody in 5% BSA solution should be kept at 4˚C and not frozen. The antibody should stay good for at least 1 month when supplemented with sodium azide.
* Weak signal:
	+ Increase amount of protein loaded
	+ Increase sensitivity of detection method (e.g. use film instead of digital imager, stronger ECL)
	+ Increase primary antibody concentration
* High background:
	+ Block with Abcam blocking buffer (ab126587) if not already doing so as it will significantly decrease background
	+ Decrease secondary antibody concentration
	+ If using film, carefully decant the membrane on the backside against a piece of Kimwipe after ECL incubation to remove excess ECL, make sure there are no dry spots and the membrane is uniformly damp when proceeding to imaging

Immunohistochemistry

* Paraformaldehyde used for fixation should always be made fresh
* Abcam blocking buffer should not be used for IHC staining of pATG16L1
* If animals do not seem to be perfusing well, readjust the needle in the heart and add more time (5min) to the total perfusion time.