Product Manual

Cellular Autophagy ELISA Kit (LC3-II Quantitation)

Catalog Number

CBA-5117 **48 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

Autophagy is a lysosomal degradation pathway for cytoplasmic material, which is activated during stress conditions such as amino acid starvation or viral infection. Mammalian cells use autophagy during short periods of starvation to degrade nonessential cellular components in order to liberate nutrients for vital biosynthetic reactions. Recent results have shown that autophagy also contributes to development, growth regulation and cancer, as well as longevity.

After induction by a stress signal such as amino acid starvation, the first step in autophagy is the formation of an autophagosome. A well published autophagosome marker protein, MAP LC3, was originally identified as a microtubule associated protein and named 'microtubule-associated-protein-light-chain-3'. LC3 is a small 16-18 kDa protein that is soluble in nonstarved cells, but becomes peripherally membrane-associated during amino acid starvation. By immunoelectron microscopy, LC3 has been shown to associate to the inner and outer limiting membranes of autophagosomes, and the membrane association is mediated by a covalent conjugation to a lipid, phosphatidylethanolamine. In Western blots, two forms of LC3 are seen, LC3-I and LC3-II. LC3-I is found in the soluble fraction, and LC3-II in the pelletable membrane fraction. Both LC3-I and LC3-II are seen in nonstarved cells, but during autophagy induction the proportion of LC3-II increases.

Cell Biolabs' Cellular Autophagy ELISA Kit (LC3-II Quantitation) is an enzyme immunoassay developed for detection and quantitation of LC3 in cultured cells. The kit utilizes a selective permeabilization procedure to remove the cytosolic pro-LC3 and LC3-I and retain the autophagosome membrane bound LC3-II (Figure 1). After fixation and permeabilization, cells containing LC3-II are probed with an anti-LC3 antibody, followed by an HRP conjugated secondary antibody. Each kit provides sufficient reagents to perform up to 48 assays.

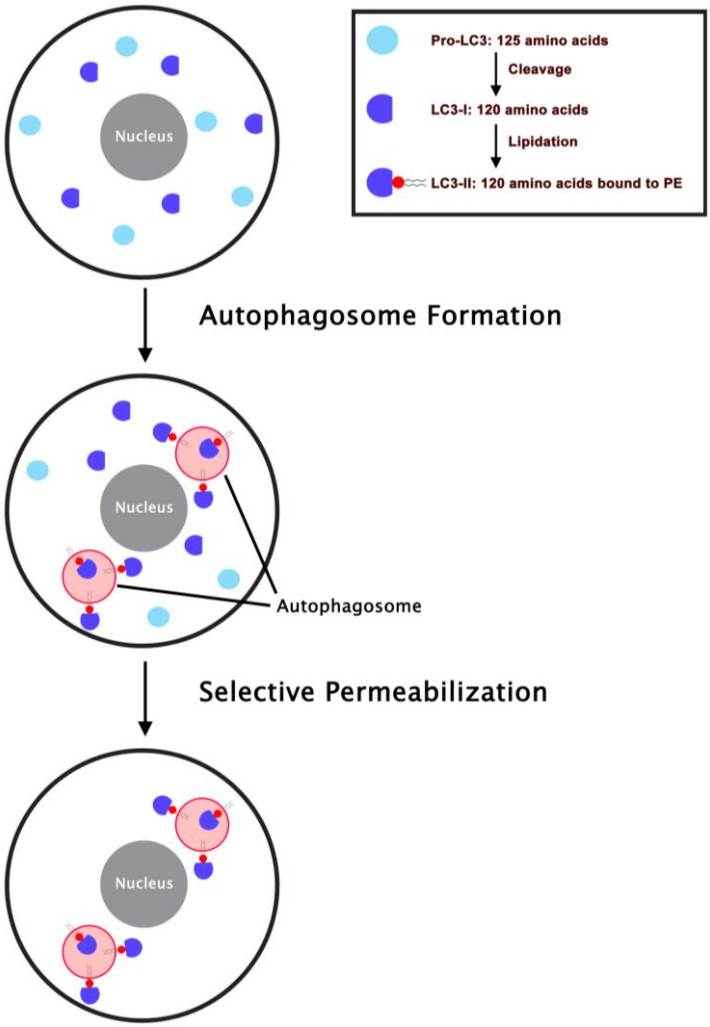

Figure 1: Processing of LC3. During autophagy, pro-LC3 undergoes C-terminal proteolysis to form LC3-I (Cytosolic). LC3-I protein conjugates to phosphatidylethanolamine (PE) and translocates to the autophagosomal membrane (LC3-II). A selective permeabilization procedure is utilized to remove cytosolic pro-LC3 /LC3-I and retain autophagosome-bound LC3-II. LC3-II is used as a specific marker of autophagy.

Related Product

1. CBA-5116: Autophagy ELISA Kit (LC3-II Quantitation)

Kit Components

1. 100X Cytosolic LC3 Removal Reagent (Part No. 51171A): One 100 µL tube.
2. 2X Fixing Solution (Part No. 51172B): One 10 mL bottle containing 8% paraformaldehyde.
3. Quenching Solution (Part No. 51173B): One 10 mL bottle.
4. Anti-LC3 Antibody (Part No. 51174C): One 10 µL tube.
5. Secondary Antibody, HRP Conjugate (1000X) (Part No. 231009): One 20 µL tube.
6. Assay Diluent (Part No. 310804): One 50 mL bottle.
7. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.
8. Substrate Solution (Part No. 310807): One 12 mL amber bottle.
9. Stop Solution (Part. No. 310808): One 12 mL bottle.
10. Cell Stain Solution (Part No. 11002): One 10 mL bottle.
11. Extraction Solution (Part No. 11003): One 10 mL bottle.

Materials Not Supplied

1. 48-well tissue culture plate
2. Cell line of interest
3. 1X PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$
4. Permeabilization Solution: 0.1% Triton X-100 in PBS

Storage

Upon receipt, store the Anti-LC3 Antibody and Secondary Antibody at -20ºC. Store all other components at 4ºC.

Preparation of Reagents

- 1X Cytosolic LC3 Removal Reagent: Warm tube to room temperature. FRESHLY prepare desired amount of 1X Cytosolic LC3-I Removal Reagent by diluting the provided 100X stock 1:100 in 1X PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. For example, add 50 µL of 100X Cytosolic LC3 Removal Reagent to 5.0 mL of 1X PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. **Do not store diluted solutions.**
- 1X Wash Buffer: Dilute the 10X Wash Buffer to 1X with deionized water. Stir to homogeneity.
- Anti-LC3 Antibody and Secondary Antibody: Immediately before use dilute the Anti-LC3 Antibody 1:1000 and Secondary Antibody 1:1000 with Assay Diluent. **Do not store diluted solutions.**

Assay Protocol

The following assay protocol is written for adherent cells in 48-well cell culture plate. Each sample should be assayed in duplicate or triplicate. The protocol can be adapted to cell suspension culture. 96-well cell culture plates are NOT recommended because of potential cell loss during the LC3-I removal and subsequent wash steps.

Note: It is recommended to include a background control where all protocol steps are followed <u>except</u> addition of the Anti-LC3 Antibody. The OD signal from this control will be subtracted from all other OD readings (see "Calculation of Results").

I. Cell Culture and Induction of Autophagy

1. Harvest and resuspend cells in culture medium at 1-5 x 10^5 cells/mL. Seed 500 µL in each well of a 48-well tissue culture plate and incubate overnight at 37ºC, 5% CO_2 (cells should be >80% confluent).
2. Wash cells once with 500 µL of 1X PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and culture them in regular medium or starvation medium such as EBSS or HBSS at 37°C for desired time to induce autophagy. Agents that inhibit or stimulate autophagy can be added directly to the cell culture.

II. LC3-I Removal, Cell Fixation and Permeabilization

1. Wash cells once with 1X PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Gently aspirate the PBS solution and add 200 µL of 1X Cytosolic LC3 Removal Reagent. Incubate at room temperature for exactly 5 minutes on an orbital shaker.
2. Immediately fix cells by directly adding 200 µL of the 2X Fixing Solution to the wells containing 1X Cytosolic LC3 Removal Reagent. Incubate 20 min at room temperature.
3. Gently aspirate the Fixing Solution and wash cells once with 500 µL of 1X PBS.
4. Permeabilize cells with 200 µL of Permeabilization Solution for 10 min. Gently wash cells once with 500 µL of 1X PBS.
5. Add 200 µL of Quenching Solution and incubate for 10 min. Gently wash cells once with 500 µL of 1X PBS.
6. Block the plate with 400 µL of Assay Diluent for 1 hour at room temperature.

III. LC3-II Detection

1. Aspirate the wells and add 200 µL of the diluted anti-LC3 antibody to each well. Incubate at room temperature for 1- 2 hours on an orbital shaker.
2. Gently wash three times with 500 µL 1X Wash Buffer per well with gentle aspiration between each wash. After the last wash, empty wells and tap plate on absorbent pad or paper towel to remove excess 1X Wash Buffer.
3. Add 200 µL of the diluted Secondary Antibody, HRP Conjugate to each well. Incubate at room temperature for 1 hour on an orbital shaker.
4. Gently wash three times according to step 2 above. Proceed immediately to the next step.
5. Warm Substrate Solution to room temperature. Add 200 µL of Substrate Solution to each well. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 5-20 minutes.

   Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.
6. Stop the enzyme reaction by adding 200 µL of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).

7. Transfer 150 µL to a 96-well microtiter plate and read absorbance of each microwell on a standard microplate reader using 450 nm as the primary wave length. Results should be read immediately (color will fade over time).

IV. Cell Number Normalization (optional)

1. Aspirate the Substrate/Stop solution from each well and wash cells once with 500 µL of 1X PBS.
2. Add 200 µL of Cell Stain Solution. Incubate for 10 minutes at room temperature.
3. Aspirate the Cell Stain Solution from the wells and discard. Gently wash each well 4-5 times with 500 µL deionized water.
4. Discard the final wash and let the wells air dry.
5. Add 200 µL of Extraction Solution per well, and then incubate 10 minutes on an orbital shaker.
6. Transfer 150 µL from each extracted sample to a 96-well microtiter plate and measure the OD 560nm in a plate reader.

Example of Results

The following figure demonstrates typical blot results of Cell Biolabs Cellular Autophagy ELISA Kit (LC3-II Quantitation). One should use the data below for reference only. This data should not be used to interpret actual results.

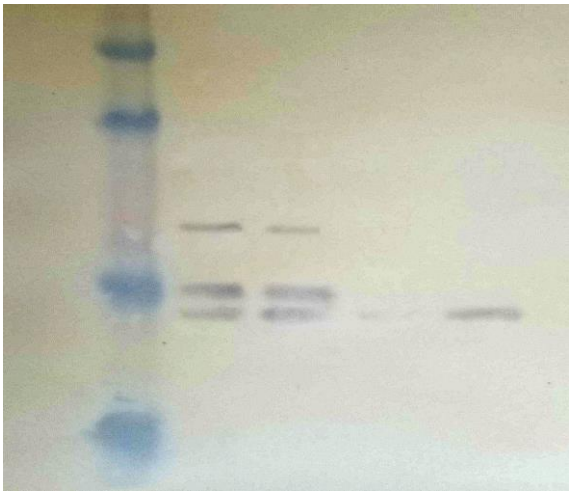

Figure 2: LC3 Removal and Immunoblotting of LC3-II. HeLa cells were treated overnight with 50 µM chloroquine. After cytosolic LC3 removal, HeLa cell lysates were prepared in 1X RIPA buffer. LC3 in HeLa lysates was detected using a polyclonal antibody against LC3.

Samples in Western Blot:

Lane 1: Prestained MW Standards
Lane 2: HeLa Control Lysate (**without** cytosolic LC3 removal)
Lane 3: Chloroquine treated HeLa Lysate (**without** cytosolic LC3 removal)
Lane 4: HeLa Control Lysate (**with** cytosolic LC3 removal)
Lane 5: Chloroquine treated HeLa Lysate (**with** cytosolic LC3 removal)

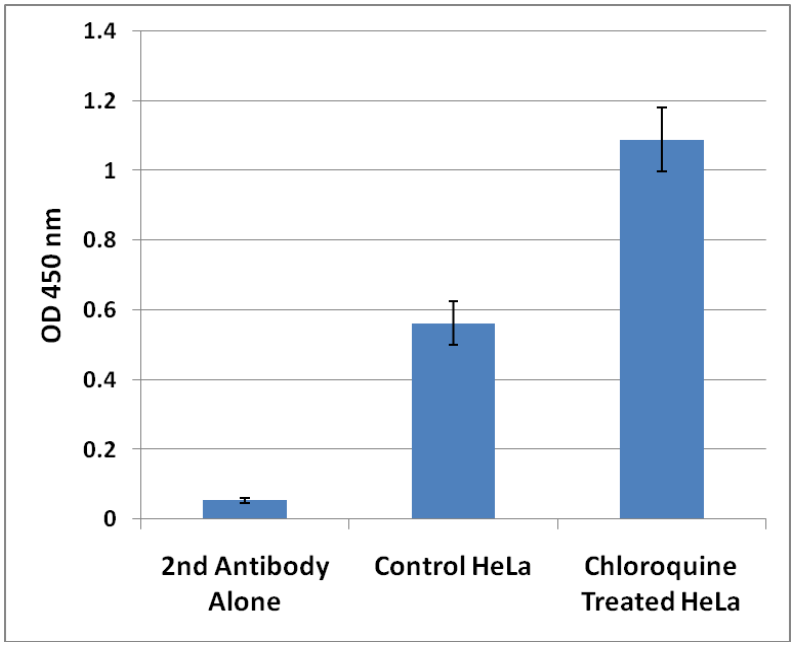

Figure 3: Cellular LC3 ELISA of Chloroquine treated HeLa Cells. HeLa cells were treated overnight with 50 µM chloroquine in a 96-well plate. After cytosolic LC3 removal, LC3-II levels in control or chloroquine treated HeLa cells were assayed as described in the Assay Protocol.

Calculation of Results

1. To correct for the background, subtract the OD of the background control well incubated in the absence of anti-LC3 antibody from each sample OD obtained in section III above:

$$\text{Net OD} = OD_{\text{sample}} - OD_{\text{background}}$$

2. (optional) Normalize each sample to cell number by dividing the Net OD (450 nm) by the cell normalization OD obtained in section IV above (560 nm).

References

1. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) *EMBO J.* **19,** 5720-5728.
2. Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., and Ohsumi, Y. (2004) *Mol. Biol. Cell* **15,** 1101-1111.

Warranty

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