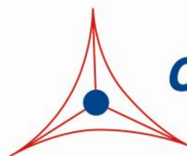
Product Manual

Autophagy ELISA Kit (LC3-II Quantitation)

Catalog Number

CBA-5116	**96 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



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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' Autophagy ELISA Kit (LC3-II Quantitation) is an enzyme immunoassay developed for detection and quantitation of LC3 in cell lysate samples. 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). The quantity of LC3-II in unknown lysate sample is determined by comparing its absorbance with that of a known LC3 standard curve. Each kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown samples.

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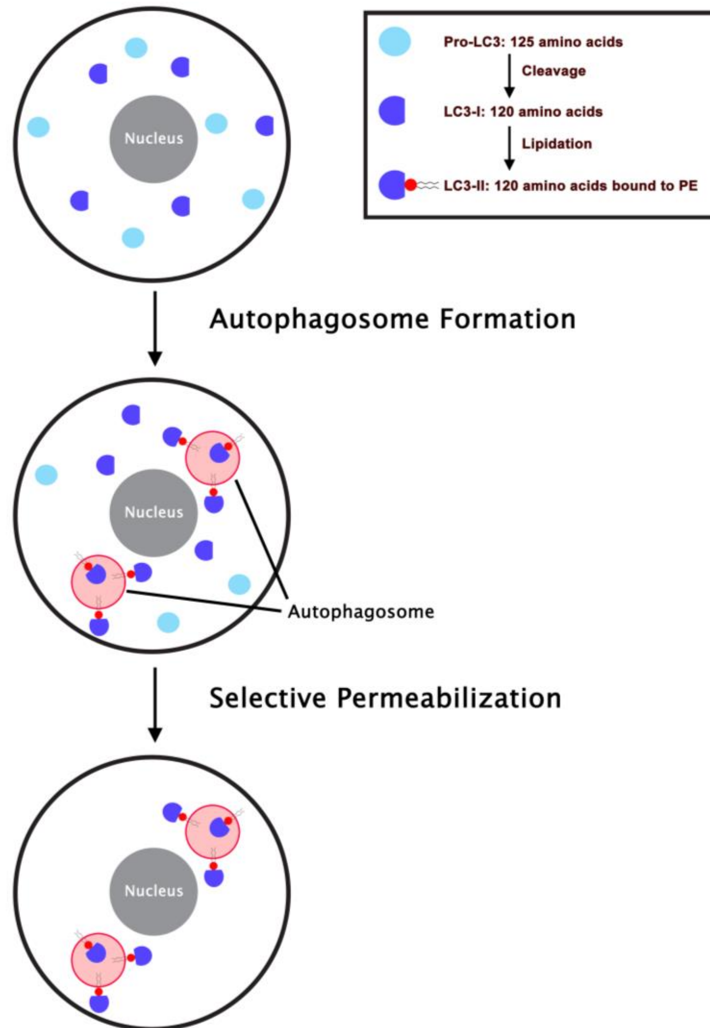

Figure 1: Processing of LC3. During autophagy, pro-LC3 undergoes C-terminal proteolysis to form LC3-I (Cytosolic). LC3-I protein conjugates to phosphatidylethanolamine (PE) and translocates to the autophagosomal membrane (LC3-II). A selective permeabilization procedure is utilized to remove cytosolic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

Related Product

1. CBA-5117: Cellular Autophagy ELISA Kit (LC3-II Quantitation)

Kit Components

Box 1 (shipped at room temperature)

1. Anti-LC3 Antibody Coated Plate (Part No. 51161B): One 96-well strip plate (8 x 12).
2. 100X Cytosolic LC3 Removal Reagent (Part No. 51151A): One 1 mL tube
3. Anti-LC3 Antibody (Part No. 51162C): One 50 µL tube of anti-LC3.
4. Secondary Antibody, HRP Conjugate (1000X) (Part No. 230003): One 20 µL vial.
5. Assay Diluent (Part No. 310804): One 50 mL bottle.
6. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.
7. Substrate Solution (Part No. 310807): One 12 mL amber bottle.
8. Stop Solution (Part. No. 310808): One 12 mL bottle.

Box 2 (shipped on blue ice packs)

1. Recombinant LC3 Standard (Part No. 51163D): One 100 µL tube of 1.0 µg/mL recombinant LC3 in TBS plus BSA.

Materials Not Supplied

1. 1X RIPA Buffer: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100 or NP-40, 1% sodium deoxycholate, 0.1% SDS containing proteinase inhibitors.
2. 1X PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$

Storage

Upon receipt, store the Recombinant LC3 Standard at -80ºC and the Anti-LC3 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 1:200 and Secondary Antibody 1:1000 with Assay Diluent. **Do not store diluted solutions.**

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Preparation of LC3 Standard

Prepare a dilution series of recombinant LC3 standards in the concentration range of 0 ng/mL to 2 ng/mL in Assay Diluent (Table 1).

Standard Tubes	1.0 µg/mL Recombinant LC3 Standard (µL)	Assay Diluent (µL)	LC3 (ng/mL)
1	10	490	20
2	250 of Tube #1	250	10
3	250 of Tube #2	250	5
4	250 of Tube #3	250	2.5
5	250 of Tube #4	250	1.25
6	0	250	0

Table 1. Preparation of samples for LC3 Standard Curve

Pro-LC3/LC3-I Removal and Cell Lysate Preparation

The following assay protocol is written for adherent cells in a 60 mm cell culture dish. Refer to the below table for the appropriate dispensing volumes of other plate formats. The protocol can be adapted to cell suspension culture.

Culture Dish	35 mm	60 mm	100 mm
1X Cytosolic LC3 Removal Reagent (mL)	1	1.5	5
PBS Wash (mL)	2	3	10
1X RIPA Buffer (mL)	0.1	0.15	0.5

Table 2: Dispensing Volumes of Different Plate Formats.

1. Culture adherent cells in 60 mm cell culture dishes to 80% confluency.
2. Wash the cells once with 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.
3. Wash cells with 3 mL of 1X PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Aspirate completely and add 1.5 mL of 1X Cytosolic LC3 Removal Reagent. Incubate at room temperature for 5 minutes on an orbital shaker.
4. Gently wash the treated cells three times with 3 mL of 1X PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$.
5. Completely remove the final PBS wash and add ice-cold 1X RIPA Buffer or desired lysis buffer to the cells (0.15 to 0.3 mL per 60 mm cell culture plate).
6. Place the culture plates on ice for 10 minutes. Detach the cells from the plates by scraping with a cell scraper. Transfer the lysates to appropriate size tubes and place on ice.

7. If nuclear lysis occurs, the cell lysates may become very viscous and difficult to pipette. If this occurs, lysates can be passed through a 27½-gauge syringe needle 3-4 times to shear the genomic DNA.
8. Clear the lysates by centrifugation for 10 minutes (12,000 x g at 4°C).
9. Collect the supernatant, determine protein concentrations by BCA assay and store lysate samples on ice for immediate use, or snap freeze and store at -80°C for future use.

ELISA Protocol

1. Add 100 µL of LC3 lysate sample or LC3 standard to the Anti-LC3 Antibody Coated Plate. Each unknown lysate sample, LC3 standard and blank should be assayed in duplicate.

   Note: we recommend using RIPA Buffer or Assay Diluent to make series of 2-fold dilutions for each unknown lysate sample.
2. Incubate at 37ºC for at least 2 hours or 4ºC overnight.
3. Wash microwell strips 3 times with 250 µL 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
4. Add 100 µL of the diluted anti-LC3 antibody to each well.
5. Incubate at room temperature for 2 hours on an orbital shaker.
6. Wash the strip wells 3 times according to step 3 above.
7. Add 100 µL of the diluted HRP conjugated secondary antibody to all wells.
8. Incubate at room temperature for 1 hour on an orbital shaker.
9. Wash the strip wells 3 times according to step 3 above. Proceed immediately to the next step.
10. Warm Substrate Solution to room temperature. Add 100 µL of Substrate Solution to each well, including the blank wells. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 2-30 minutes.

    Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.
11. Stop the enzyme reaction by adding 100 µL of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).
12. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.

Example of Results

The following figure demonstrates typical blot results of Cell Biolabs 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.

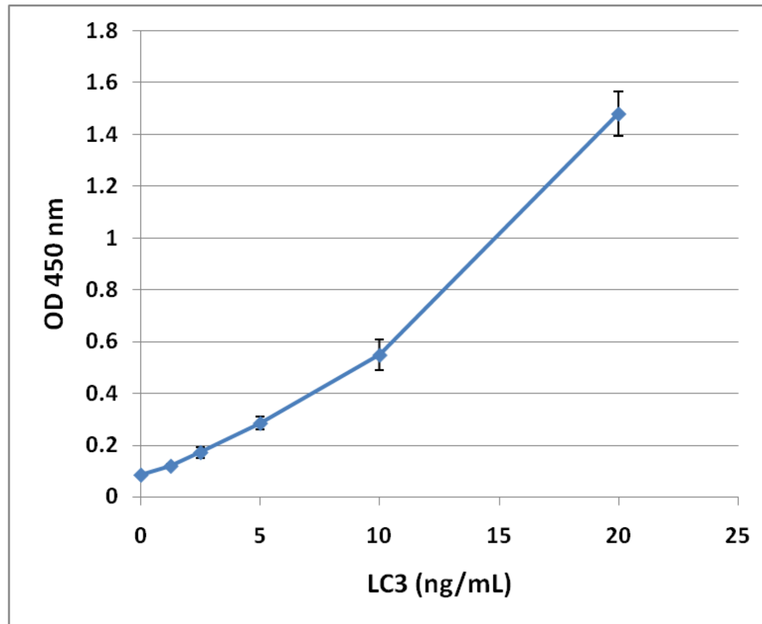

Figure 2: LC3 ELISA Standard Curve.

Figure 3: 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.

<u>**Samples in Western Blot:**</u>

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)

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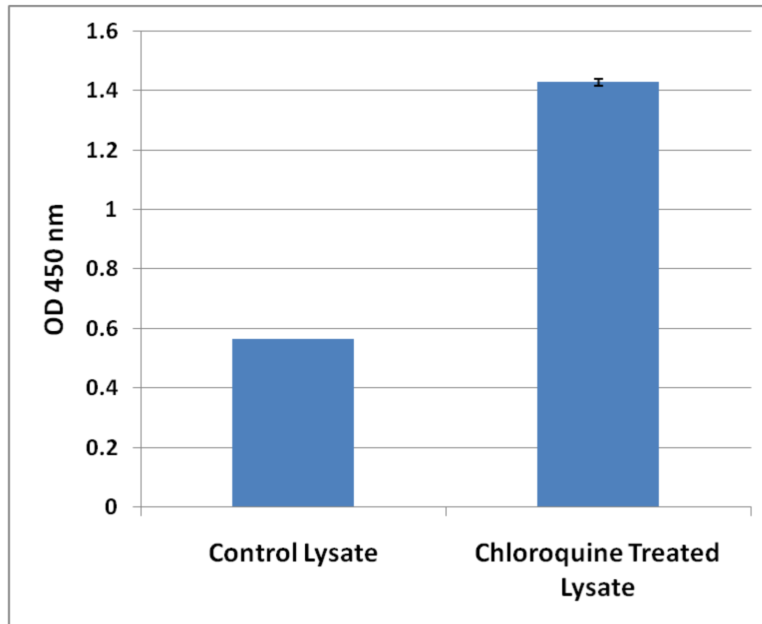

Figure 4: ELISA testing of LC3 in Chloroquine treated HeLa Cells. HeLa cells were treated overnight with 50 µM chloroquine. After cytosolic LC3 removal, HeLa cell lysates were prepared in 1X RIPA buffer. LC3-II levels in 15 µg of HeLa lysates were assayed as described in the Assay Protocol.

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.

Recent Product Citation

Awad, A.M. et al. (2023). Ameliorative effect of montelukast against STZ induced diabetic nephropathy: targeting HMGB1, TLR4, NF-κB, NLRP3 inflammasome, and autophagy pathways. *Inflammopharmacology*. doi: 10.1007/s10787-023-01301-1.

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