LC3-II Enrichment Kit (Western Blot)

Catalog No. 17-10232

15 reactions

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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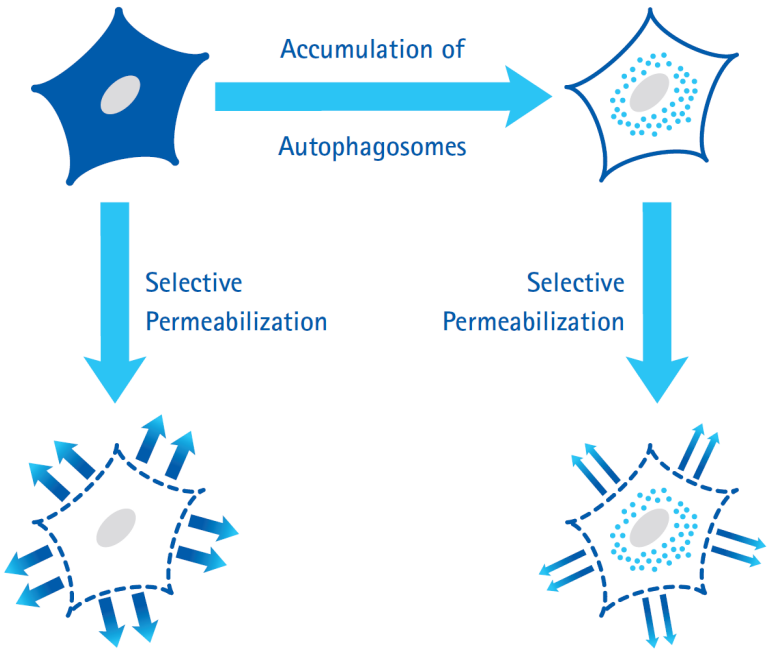
Introduction

Autophagy, a degradative pathway that provides recycled nutrients to cells under stress, plays both protective and deleterious roles in many diseases, including cancer, neurodegeneration, and infections[1]. Members of the LC3 family play a key role in the maturation of the autophagosome, the central organelle of autophagy. LC3 precursors are proteolytically processed to form LC3-I, which is diffusely distributed in the cytosol. Upon initiation of autophagy, the C-terminal glycine of LC3-I is modified by addition of a phosphatidylethanolamine (PE) to form LC3-II, which translocates rapidly to nascent autophagosomes in a punctate distribution[2].

Given the pronounced changes in biochemical properties and subcellular localization of LC3 upon initiation of autophagy, detection of LC3 by various means has become the gold standard method for estimating autophagosome quantity within the cell[3]. However, detecting and interpreting the relative amounts of LC3-I and LC3-II in standard assay methods can be complicated. When LC3 is immunochemically detected by Western Blotting following SDS-polyacrylamide gel electrophoresis, LC3-II typically appears as a slightly lower band than the 18 kDa LC3-I band as a result of the greater hydrophobicity of LC3-II. **However, in some instances, the LC3-I and LC3-II bands are incompletely resolved, which complicates analysis.** Also, even when LC3-I and LC3-II are adequately separated on an immunoblot, changes in the relative ratios of the isoforms may not be directly proportional to autophagosome quantity, due to differential immunoreactivity of the isoforms. Finally, there is confusion about whether the best indicator of autophagy is 1) absolute quantity of LC3-II, 2) the relative ratio of LC3-II to LC3-I alone, or 3) the relative ratio of LC3-I to the sum of LC3-I and LC3-II. A review solely devoted to interpretation of LC3 immunoblotting concludes that the absolute quantity of LC3-II is the best indicator of autophagy[4].

EMD Millipore's LC3-II Enrichment Kit (Western Blot) enables sensitive and accurate quantification of autophagosome density by utilizing a selective permeabilization procedure that removes cytosolic LC3-I and retains autophagosome-bound LC3-II. This procedure reveals quantities of LC3-II, without interference from LC3-I, by Western blotting analysis. The kit also includes a lysosomal inhibitor, to prevent degradation of LC3-II that occurs upon fusion of the autophagosome with the lysosome. In addition, an antibody against a mitochondrial outer membrane protein TOMM22 is included for use as a control for specificity of permeabilization, to ensure that organelles remain intact after treatment.



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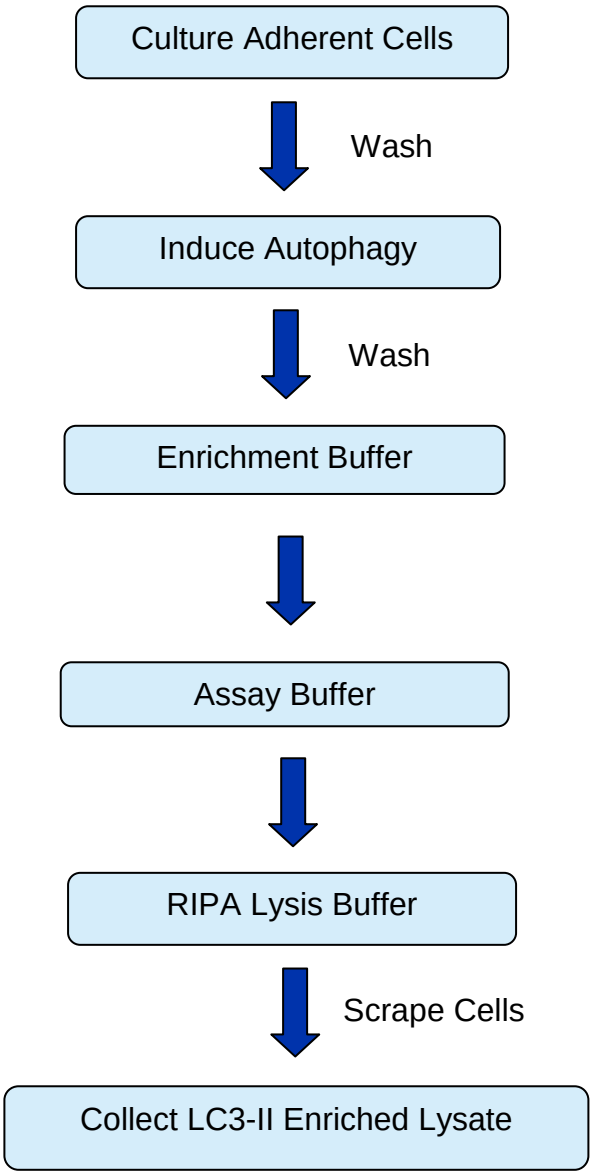
Protocol Flow Chart:



Kit Components

LC3-II Enrichment (17-10232-1)
Store at 2-8º C

1. Autophagy Reagent A: (Part No. CS208212). One vial, lyophilized.
2. 10x Enrichment Buffer: (Part No. CS210560). One bottle containing 10 mL 10x Enrichment Buffer.
3. 5x Assay Buffer: (Part No. CS210561). One bottle containing 15 mL 5x Assay Buffer.
4. 10x RIPA Lysis Buffer: (Part No. CS210559). One bottle containing 1 mL 10x RIPA Lysis Buffer.

Western Blot Detection (17-10232-2)
Store at -20º C

1. Protease Inhibitor Cocktail: (Part No. CS210562). One vial containing 20 µL of 1000x Protease Inhibitor Cocktail.
2. Anti-LC3 antibody: (Part No. CS210563). One vial containing 20 µL of 1000x anti-LC3 rabbit monoclonal antibody.
3. Anti-TOMM22 antibody: (Part No. CS210516). One vial containing 20 µL of 1000x anti-TOMM22 mouse monoclonal antibody.
4. Goat anti-Mouse IgG HRP, Secondary Antibody: (Part No. 2003482). One vial containing 30 µL of 2000X goat anti-mouse HRP conjugate.
5. Goat anti-Rabbit IgG HRP, Secondary Antibody: (Part No. 90276). One vial containing 30 µL of 2000X goat anti-rabbit HRP conjugate.

***Note:** The quantity of buffers provided is sufficient for preparation of lysates from **fifteen (15) x 100 mm cell culture dishes**. Dishes of different sizes may be used, with volumes adjusted as described in **Table 1** in the Appendix.

Materials Not Supplied

1. Pipettors and tips capable of accurately measuring 1-1000 µL
2. Graduated serological pipettes
3. Microcentrifuge tubes
4. Mechanical vortex
5. Rocker
6. Distilled or deionized water
7. 1x Dulbecco's Phosphate Buffered Saline (D-PBS)
8. 1x Tris-buffered saline containing 0.05% Tween-20 (TBST)
9. 3% Non-fat dry milk diluted in TBST (TBST-MILK)
10. Earle's Balanced Salt Solution (EBSS)

Storage

Maintain the unopened kit boxes (17-10232-1 and 17-10232-2) at the indicated temperatures. The kit may be used for up to **4 months** after receipt. Avoid repeated freeze/thaw cycles, and aliquot if necessary.

Upon receipt, store 10x RIPA Lysis Buffer (Cat No. CS210559) at **room temperature**.

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.
- **Safety Warnings and Precautions:** This kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

Technical Notes

- All kit reagents should be rapidly thawed just prior to use, keep on ice until use.
- Do not mix or interchange reagent from various kit lots.

Reagents Preparation

1. **Autophagy Reagent A:** This material is supplied in a lyophilized vial. Prior to use, reconstitute the contents of the vial in 250 µL Milli-Q or deionized water to make 100 mM stock solution. Aliquot and store at -20ºC. Avoid repeated freezing and thawing.
2. **1x Enrichment Buffer:** Prepare 90 mL of 1x Enrichment Buffer by adding 9 mL of 10x Enrichment Buffer (Part No. CS210560) to 81 mL of Milli-Q or deionized water. Store at 2-8ºC.
3. **1x Assay Buffer:** Prepare 75 mL of 1x Assay Buffer by adding 15 mL 5x Assay Buffer (Part No. CS210561) to 60 mL of Milli-Q or deionized water. Store at 2-8ºC.
4. **1x RIPA Lysis Buffer:** Prepare 5 mL of 1x RIPA Lysis Buffer by adding 0.5 mL 10x RIPA Lysis Buffer (Part No. CS210559) to 4.49 mL Milli-Q or deionized water. Add 5 µL of Protease Inhibitor Cocktail (Part No. CS210562) and vortex to mix. Aliquot and store at -20ºC.

Protocol

LC3-II Enrichment:

1. Culture adherent cells to approximately 80-90% confluency in a 100 mm cell culture dish.

   Note: The quantity of buffers provided is sufficient for preparation of lysates from fifteen (15) x 100 mm cell culture dishes. Dishes of different sizes may be used, with volumes adjusted as described in Table 1 in the Appendix.

2. Once cells have reached the desired confluency, gently wash cells twice with 10 mL of EBSS. Aspirate completely after each wash.
3. The following cell treatments are recommended as controls to include alongside experimental treatments:
   a. Label one dish as “untreated” and leave in complete medium.
   b. Label a second dish as “treated” and add 10 mL of EBSS + 100 uM of Autophagy Reagent A.

   Note: Users may wish to perform experimental treatments in the presence and absence of Autophagy Reagent A, as comparison of LC3-II in the presence or absence of this reagent provides an *estimate* of autophagic flux.

4. Incubate both flasks in a humidified incubator at 37ºC for the desired time interval.

   Note: Increased LC3-II may be observed within 15 minutes of treatment, and reaches maximum levels in 2-4 hrs (**see Figure 1**).

5. Aspirate media and wash all dishes with 10 mL 1x D-PBS, twice.

6. Add 5 mL 1x Enrichment Buffer to dishes under enrichment. Place plates on rocker, and incubate at room temperature for 5 minutes. Leave dishes that are not under enrichment in 1x D-PBS.
7. Discard buffers from all dishes.
8. Add 5 mL 1x Assay Buffer to all dishes and aspirate completely.
9. Add 150 µL 1x RIPA Lysis Buffer to dishes and incubate for 5 minutes.
10. Scrape cells in Lysis Buffer and collect in a 1.5 mL microcentrifuge tube. Incubate tubes on ice for 10 minutes with periodic vortexing.
11. Centrifuge samples at 12,000 rpm for 10 minutes. Collect supernatants for analysis.
12. Determine protein concentrations of the supernatants by A_{280}.

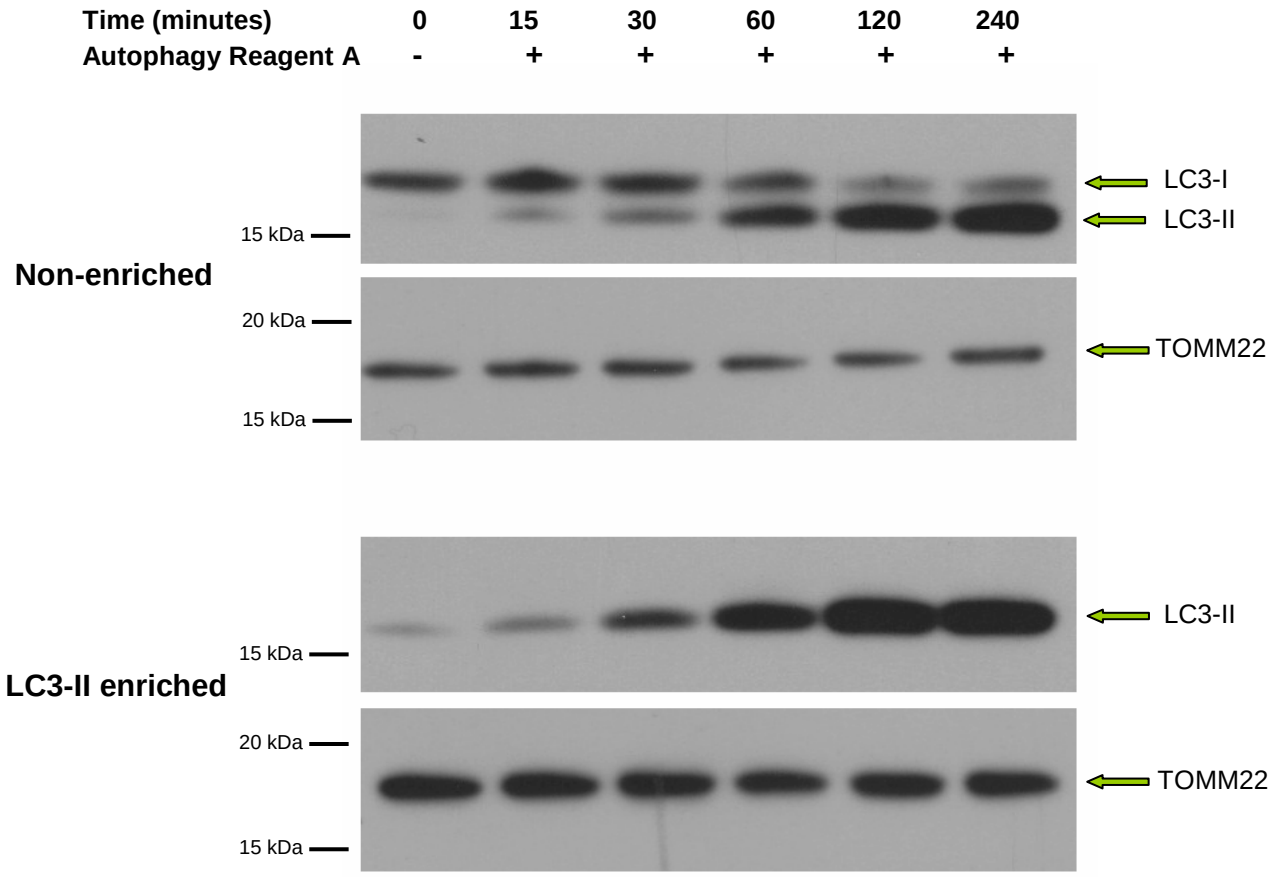
Western Blot Detection:

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on collected lysates, on duplicate gels.
2. Transfer proteins to PVDF membranes. Wash blotted membranes twice with water.
3. Block the blots in freshly prepared TBST-MILK, for 1 hour at room temperature with constant agitation.
4. Incubate the separate blots with
    a. 1:1,000 dilution of anti-LC3 antibody, diluted in TBST-MILK.
    b. 1:1,000 dilution of anti-TOMM22 antibody, diluted in TBST-MILK

    for 2-3 hours at room temperature (or alternately overnight at 4ºC) with agitation.

    Note: Sufficient antibody concentrates are supplied for preparing 20 mL of primary antibody incubation solution. Typically this volume would be sufficient for probing two 2.75” x 3.5” blots.
5. Wash the blots once with TBST for 5 minutes followed by washing twice with distilled water for 5 minutes each.
6. Incubate the indicated blots with the following secondary antibodies for 45 minutes at room temperature with agitation:
    a. Anti-LC3 blot: Goat-anti-rabbit, HRP secondary antibody in TBST-MILK (Part No. 90276)
    b. Anti-TOMM22 blot: Goat-anti-mouse, HRP secondary antibody in TBST-MILK (Part No. 2003482)
7. Wash the blots once with TBST for 5 minutes, then wash twice with distilled water for 5 minutes each. Develop the blots with the detection method of choice.

Representative Data



Representative Western Blot Detection Data

Figure 1. Western Blot of non-enriched lysate and LC3-II-enriched protein fractions from HeLa cells prepared with the LC3-II Enrichment Kit (Western Blot) (Catalogue No. 17-10232). Proteins were subjected to SDS-PAGE and were transferred to a PVDF membrane. The membrane was probed with primary anti-LC3 and anti-TOMM22, followed by secondary antibodies. The blot was developed by enhanced chemiluminescence. Immunoblotting results of non-enriched lysates indicate the LC3-I signal decreases over time after induced autophagy, as the LC3-II signal increases.

After enrichment, the LC3-I signal is no longer detectable and the LC3-II signal is retained.

Appendix

Table 1: Buffer Volumes & Dish Size

Volumes of buffers used in kit for different sized cell culture vessels.
All volumes listed in milliliters (mL).

Buffer used	Step	60mm dish	100mm dish	150mm dish
EBSS	2	5	10	15
EBSS + Autophagy Reagent A	3	5	10	15
1x D-PBS	4	5	10	15
1x Autophagosome Enrichment Buffer	5	3	5	7.5
1x Assay Buffer	7	3	5	7.5
1x RIPA Lysis Buffer	8	0.1	0.15	0.3

Troubleshooting Guide

Problem	Potential Cause	Experimental Suggestions
No signal or weak signal for TOMM22	Loss of cells during washes	Some cells adhere more strongly than others. Check cells using microscope during protocol to be certain that cells are retained.
	Primary antibody concentration too low	Titrate antibody concentration.
	Antibody not compatible with species of cell type	The antibodies have been validated with human, mouse and rat cell lines. Results with other species may vary.
No signal for LC3-II where expected	Loss of cells during washes, primary antibody concentration too low, or antibody not compatible with species of cell type	See above.
	Degradation of autophagosomes	Ensure that Autophagy Reagent A is included to inhibit lysosomal degradation of LC3-II
LC3-I incompletely depleted	Incomplete permeabilization during enrichment	Increase incubation time or volume of Enrichment Buffer.

References

1. Levine, B. and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell* 132: 27-42.

2. Kabeya, Y. *et al.* (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* 19: 5720-5728.

3. Klionsky, D. J. *et al.* (2008). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 4: 151-175.

4. Mizushima, N. and Yoshimori, T. (2007). How to interpret LC3 immunoblotting. *Autophagy* 3: 542-545.

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