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MBL
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MONOCLONAL ANTIBODY

Anti-LC3 mAb

Code No.	Clone	Subclass	Quantity	Concentration
M152-3	4E12	Mouse IgG1 κ	100 µL	2 mg/mL

BACKGROUND: Macroautophagy mediates the bulk degradation of cytoplasmic components. These components are delivered to lysosomes via autophagosomes. The rat microtubule-associated protein 1 light chain 3 (LC3), a homologue of yeast Atg8 (Aut7/Apg8), localizes to autophagosomal membranes after post-translational modifications. The C-terminal fragment of LC3 is cleaved immediately following synthesis to yield a cytosolic form called LC3-I. A subpopulation of LC3-I is further converted to an autophagosome-associating form, LC3-II. This antibody can detect both forms of LC3.

SOURCE: This antibody was purified from hybridoma (clone 4E12) supernatant using protein A agarose. This hybridoma was established by fusion of mouse myeloma cell P3U1 with C3H mouse lymphocyte immunized with the recombinant human LC3 [MAP1LC3B (1-120 aa)].

FORMULATION: 200 µg IgG in 100 µL volume of PBS containing 50% glycerol, pH 7.2. No preservative is contained.

STORAGE: This antibody solution is stable for one year from the date of purchase when stored at -20°C.

REACTIVITY: This antibody reacts with LC3 (MAP1LC3A, B) on Immunocytochemistry, Western blotting, Immunoprecipitation, and Flow cytometry.

REFERENCES:

1) Kabeya, Y., *et al*., *J. Cell Sci.* **117**, 2805-2812 (2004)
2) Mizushima, N., *et al*., *Mol. Biol. Cell* **15**, 1101-1111 (2004)
3) Mizushima, N., *et al*., *J. Cell Biol.* **152**, 657-667 (2001)
4) Kabeya, Y., *et al*., *EMBO J.* **19**, 5720-5728 (2000)

SPECIES CROSS REACTIVITY:

Species	Human	Mouse	Rat	Hamster
Cells	HeLa	NIH/3T3, MEF	PC12	CHO
Reactivity on WB	+	+	+	+

APPLICATIONS:

Western blotting; Not recommended
MBL code no. M186-3, M186-7 and PM036 are more suitable for Western blotting*.
Immunoprecipitation; 5 µg/300 µL of cell extract from 1 x 10^7 cells
Immunohistochemistry; reported*
Immunocytochemistry; 40 µg/mL
Flow cytometry; 40 µg/mL (final concentration)
Immuno-electron microscopy; 20 µg/mL
Image-based flow cytometry; reported*

*For further information, please visit our web site at https://ruo.mbl.co.jp/.

Detailed procedure is provided in the following **PROTOCOLS**.

INTENDED USE:

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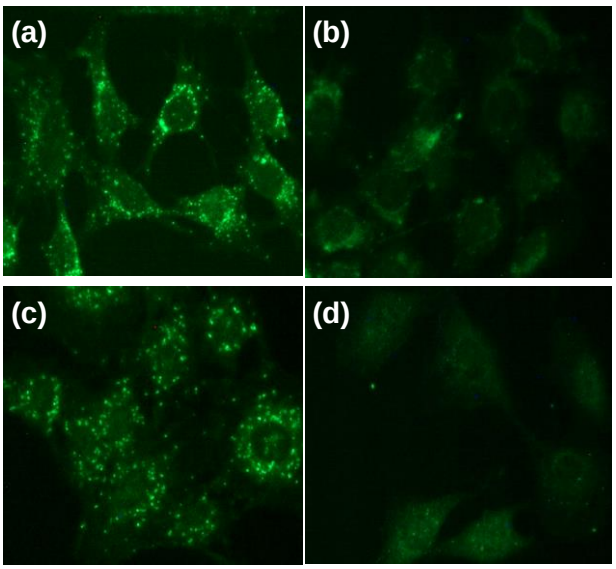


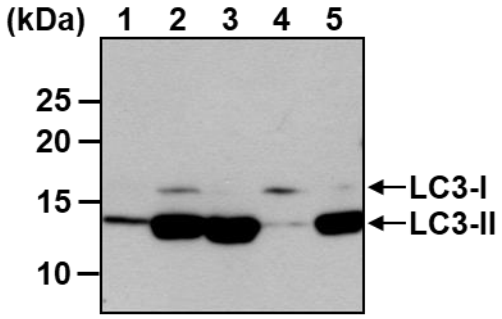
Immunocytochemical detection of LC3 in 4% PFA fixed starved MEF (a), nutrient MEF (b), starved NRK (normal rat kidney cell line) (c) and nutrient NRK (d) with M152-3.

PROTOCOLS:

Immunocytochemistry

1) Spread the cells in the nutrient condition on a glass slide, then incubate in a CO_2 incubator for one night.
2) Remove the culture supernatant by careful aspiration.
3) To obtain serum-starved conditions, culture the cells with Hank's solution or DMEM for 2-4 hours at 37°C.
4) Fix the cells by immersing the slide in 4% paraformaldehyde (PFA)/PBS for 10 minutes at room temperature (20~25°C).
5) Prepare a wash container such as a 500 mL beaker with a magnetic stirrer. Then wash the fixed cells on the glass slide by soaking the slide with a plenty of PBS in the wash container for 5 minutes. Take care not to touch the cells. Repeat another wash once more.
6) Immerse the slide in 100 μg/mL of Digitonin for 10 minutes at room temperature.
7) Wash the slide in a plenty of PBS as in the step 5).
8) Add the primary antibody diluted with PBS as suggested in the **APPLICATIONS** onto the cells and incubate for 1 hour at room temperature. (Optimization of antibody concentration or incubation condition is recommended if necessary.)
9) Wash the slide in a plenty of PBS as in the step 5).
10) Add FITC-conjugated anti-mouse IgG antibody diluted with PBS onto the cells. Incubate for 30 minutes at room temperature. Keep out light by aluminum foil.
11) Wash the slide in a plenty of PBS as in the step 5).
12) Wipe excess liquid from slide but take care not to touch the cells. Never leave the cells to dry.
13) Promptly add mounting medium onto the slide, then put a cover slip on it.

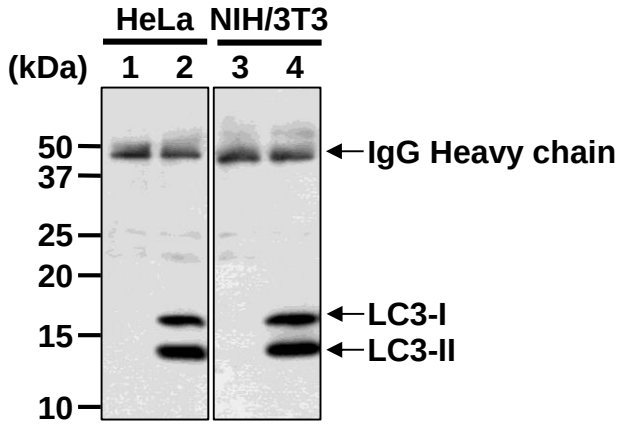
(Positive controls for Immunocytochemistry; MEF and NRK)



Western blotting analysis of LC3 expression in HeLa (1), NIH/3T3 (2), MEF (3), PC12 (4) and CHO (5) using M152-3.

SDS-PAGE & Western blotting

1) Wash the $1x10^7$ cells 3 times with PBS and suspend with 1 mL of Laemmli's sample buffer.
2) Boil the samples for 2 minutes and centrifuge. Load 10 μL of sample per lane on a 1-mm-thick SDS-polyacrylamide gel (15% acrylamide) and carry out electrophoresis.
3) Blot the protein to a polyvinylidene difluoride (PVDF) membrane at 1 mA/cm^2 for 1 hour in a semi-dry transfer system (Transfer Buffer: 25 mM Tris, 190 mM glycine, 20% Methanol). See the manufacture's manual for precise transfer procedure.
4) To reduce nonspecific binding, place the membrane in 10% skimmed milk (in PBS, pH 7.2) overnight at 4°C.
5) Wash the membrane with PBS-T [0.05% Tween-20 in PBS] (5 minutes x 3).
6) Incubate the membrane with primary antibody diluted with PBS, pH 7.2 containing 1% skimmed milk as suggested in the **APPLICATIONS** for 1 hour at room temperature. (The concentration of antibody will depend on the conditions.)
7) Wash the membrane with PBS-T (5 minutes x 3).
8) Incubate the membrane with the 1:10,000 anti-IgG (Mouse) pAb-HRP (MBL; code no. 330) diluted with 1% skimmed milk (in PBS, pH 7.2) for 1 hour at room temperature.
9) Wash the membrane with PBS-T (5 minutes x 3).
10) Wipe excess buffer on the membrane, then incubate it with appropriate chemiluminescence reagent for 1 minute. Remove extra reagent from the membrane by dabbing with paper towel, and seal it in plastic wrap.
11) Expose to an X-ray film in a dark room for 10 minutes. Develop the film as usual. The condition for exposure and development may vary.



Immunoprecipitation of LC3 from HeLa cells or NIH/3T3 with isotype control (1, 3) or M152-3 (2, 4). *After immunoprecipitated with the antibody, immunocomplex was resolved on SDS-PAGE and immunoblotted with anti-LC3 (MBL; code no. PM036).*

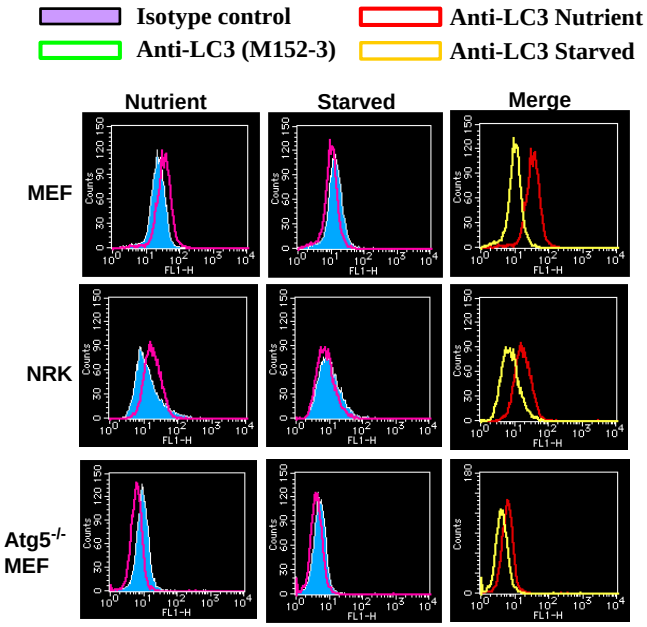
(Positive controls for Western blotting; HeLa, NIH/3T3, MEF, PC12 and CHO)

Immunoprecipitation

1) Wash the cells 3 times with PBS and suspend with 10 volumes of cold Lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% NP-40] containing appropriate protease inhibitors. Incubate it at 4°C with rotating for 30 minutes, then sonicate briefly (up to 10 seconds).

2) Centrifuge the tube at 12,000 x g for 10 minutes at 4°C and transfer the supernatant to another tube.
3) Add primary antibody as suggested in the **APPLICATIONS** into 300 µL of the supernatant. Mix well and incubate with gentle agitation for 30-120 minutes at 4°C.
4) Add 20 µL of 50% protein A agarose beads resuspended in the cold Lysis buffer. Mix well and incubate with gentle agitation for 60 minutes at 4°C.
5) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
6) Resuspend the beads with the cold Lysis buffer.
7) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
8) Repeat steps 6)-7) 3-5 times.
9) Resuspend the beads in 20 µL of Laemmli's sample buffer, boil for 3-5 minutes, and centrifuge for 5 minutes. Use 20 µL/lane for the SDS-PAGE analysis.
(See **SDS-PAGE & Western blotting**.)

(Positive controls for Immunoprecipitation; HeLa and NIH/3T3)



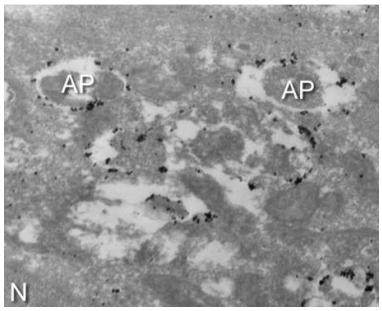
Flow cytometric analysis of LC3 expression in nutrient or starved of MEF, NRK and Atg5$^{-/-}$MEF. *Fluorescence intensity of LC3 on starved cells was reduced.*
Atg5$^{-/-}$ MEF was provided by Dr.Mizushima M.D. Ph.D.

References
・Shvets, E., and Elazar, Z., *Methods Enzymol*, **452**, 131-141 (2009)
・Shvets, E., *et al.*, *Autophagy* **4**, 621-628 (2008)

Flow cytometric analysis for adherent cells

We usually use Fisher tubes or equivalents as reaction tubes for all steps after 4).

1) To obtain serum-starved conditions, culture the cells with Hank's solution or DMEM for 4 hours at 37°C.
2) Detach the cells from culture dish by trypsinization. *Excessive trypsinization may reduce the antigenecity.
3) Wash the cells with PBS.
4) Fix the cells with 4% paraformaldehyde (PFA)/PBS for 15 minutes at room temperature (20~25°C). Wash the cells 2 times with PBS.
5) Permeabilize the cells with 100 µg/mL of Digitonin for 15 minutes at room temperature.
6) Wash the cells twice with PBS.
7) Resuspend the cells with PBS (5 x 10^6 cells/mL).
8) Add 50 µL of the cell suspension into each tube, and centrifuge at 500 x g for 1 minute at room temperature (20~25°C). Remove supernatant by careful aspiration.
9) Add 40 µL of the primary antibody at the concentration as suggested in the **APPLICATIONS** diluted in the washing buffer [PBS containing 2% fetal calf serum (FCS) and 0.09% NaN_3]. Mix well and incubate for 30 minutes at room temperature.
*Azide may react with copper or lead in plumbing system to form explosive metal azides. Therefore, always flush plenty of water when disposing materials containing azide into drain.
10) Add 1 mL of the washing buffer followed by centrifugation at 500 x g for 1 minute at room temperature. Remove supernatant by careful aspiration.
11) Add FITC-conjugated anti-mouse IgG antibody diluted with the washing buffer. Mix well and incubate for 15 minutes at room temperature.
12) Add 1 mL of the washing buffer followed by centrifugation at 500 x g for 1 minute at room temperature. Remove supernatant by careful aspiration.
13) Resuspend the cells with 500 µL of the washing buffer and analyze by a flow cytometer.



Immuno-electron microscopy detection of LC3
Cell: starved MEF
AP: autophagosome, N: nucleus
This data was provided by Ms. Sakamaki and Dr. Mizushima, Tokyo Medical and Dental University.

Immuno-electron microscopy

1) Spread the cells on Cell Desk (SUMITOMO BAKELITE; code no. MS-92132Z), then incubate in a CO_2 incubator.
2) Remove the culture supernatant by careful aspiration.
3) To obtain serum-starved conditions, culture the cells with Hank's solution or DMEM for 2-4 hours at 37°C.
4) Fix the cells with 4% paraformaldehyde (PFA)/0.1 M phosphate buffer (pH 7.4) for 2 hours at 4°C on Cell Desk.
5) Wash the Cell Desk with 0.1 M phosphate buffer (pH 7.4) for 5 minutes at 4°C. Wipe excess liquid by using a filtration paper.

6) Permeabilize the cells with 14% glycerol/35% sucrose in 0.1 M phosphate buffer (pH 7.4) for 15 seconds at 4°C.
7) Freeze once by liquid nitrogen for 15 seconds, and then thaw on ice. Following all of procedures were performed on ice.
8) Wash the Cell Desk with ice-chilled 0.1 M phosphate buffer (pH 7.4).
9) Block the cells with 0.05% silver-blocking solution [10% normal goat serum, 10% fetal calf serum, 0.1% cold water fish gelatin, and 0.005% saponin in 0.1 M phosphate buffer (pH 7.4), filtrated] for 30 minutes under agitated condition.
10) Incubate the cells with primary antibody as suggested in the **APPLICATIONS** diluted with silver-blocking solution overnight at 4°C.
11) Wash the Cell Desk with ice cold 0.05% saponin in 0.1 M phosphate buffer (pH 7.4) for 10 minutes at 4°C under agitated condition. Repeat step11) 5 times.
12) Incubate the cells with 1:100 anti-mouse IgG, Fab' fragment conjugated with NANOGOLD® (Nanoprobes; code no. 2002) diluted with silver-blocking solution for 2 hours at room temperature (20-25°C).
13) Wash the Cell Desk with ice-chilled 0.05% Saponin in 0.1 M Phosphate buffer (pH 7.4) for 10 minutes at 4°C under agitated condition. Repeat another fifth time.
14) Wash the Cell Desk with ice chilled 0.1 M Phosphate buffer (pH 7.4) for 10 minutes at 4°C under agitated condition.
15) Fix the cells with 1% gultaraldehyde in 0.1 M Phosphate buffer (pH 7.4) for 10 minutes at 4°C under agitated condition.
16) Wash the Cell Desk with 50 mM Glycine phosphate buffer for 3 minutes under agitated condition. Repeat another 2 times.
17) Wash the Cell Desk with ice cold Milli-Q water for 1 minute under agitated condition.
18) Treat with HQ SILVER™ Enhancement Kit (Nanoprobes; code no. 2012) in dark. Briefly described as bellow.
    Dispensed initiator (A) into a clean tube, added moderator (B) and mix thoroughly without bubbles, then added activator (C) and mixed thoroughly again without bubbles to prepare the reagent. Develop for appropriate time (~6 minutes) at room temperature.
19) Wash the Cell Desk with ice cold Milli-Q water. Repeat another 2 times.
20) Fix the cells with 0.3% OsO4 in 0.1 M phosphate buffer (pH 7.4) for 15 minutes at 4°C.
21) Wash the Cell Desk with ice cold 0.1 M phosphate buffer (pH 7.4) for 5 minutes at 4°C. Repeat another 2 times.
22) Dehydrate with 15, 30, 50 and 70% ethanol for 10 minutes at 4°C each.
23) Stain with 2% uranyl acetate in 70% ethanol for 1-2 hours at 4°C under agitated condition.
24) Dehydrate with 70, 80, 90, 95 and 99.5% ethanol for 10 minutes at 4°C each. Then dehydrate with 100% ethanol for 10 minutes at room temperature 3 times.
25) Soak with propylene oxide for 15 minutes at room temperature. Repeat another once treatment.
26) Soak into propylene oxide:Epon812 (1:1) for 1-2 hours at room temperature under agitated condition.
27) Soak into propylene oxide:Epon812 (1:3) for 1 hour or overnight at room temperature under agitated condition.
28) Soak into Epon812 for 2-6 hours at room temperature under agitated condition.
29) The resin was polymerized in temperature-controlled incubator for 2 days at 60°C.
30) 60 nm ultra-thin sections were prepared.
31) Sections were examined with a transmission electron microscope H-7100 (Hitachi).

(Positive control for Immuno-electron microscopy; MEF)

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