

MONOCLONAL ANTIBODY

Anti-LC3 mAb

Code No.	Clone	Subclass	Quantity	Concentration
M152-3	4E12	Mouse IgG1 κ	100 μ L	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 μ g IgG in 100 μ L 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 and Immunoprecipitation.

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:

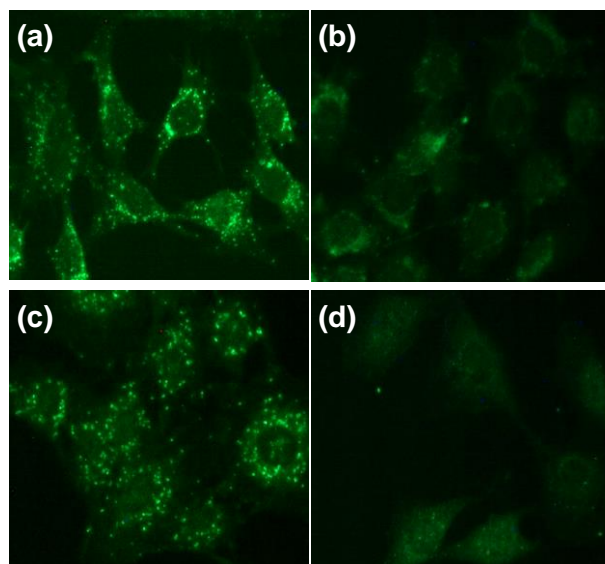
- Western blotting; Not recommended
MBL code no. M186-3, M186-7 and PM036 are more suitable for Western blotting*.
- Immunoprecipitation; 5 μ g/300 μ L of cell extract from 1×10^7 cells
- Immunohistochemistry; reported*
- Immunocytochemistry; 40 μ g/mL
- Flow cytometry; 40 μ g/mL (final concentration)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

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

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

INTENDED USE:

For Research Use Only. Not for use in diagnostic procedures.



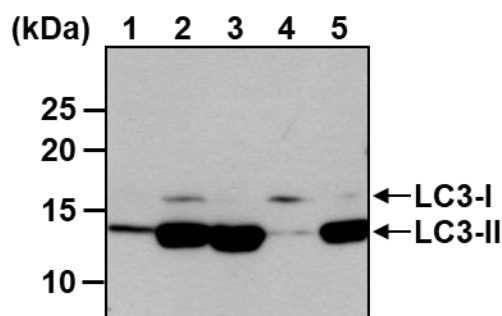
Immunocytochemical detection of LC3 in 4% PFA fixed starved MEF (a), nutrient MEF (b), starved NRK (normal rat kidney cell line) (c) and nutrient NRK (d) with M152-3.

PROTOCOLS:

Immunocytochemistry

- 1) Spread the cells in the nutrient condition on a glass slide, then incubate in a CO₂ 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.

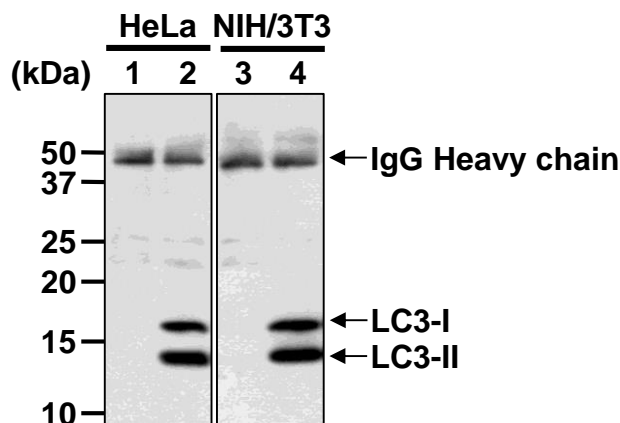
SDS-PAGE & Western blotting

- 1) Wash the 1x10⁷ 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/cm² 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 times).
- 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 times).
- 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 times).
- 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.

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



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).

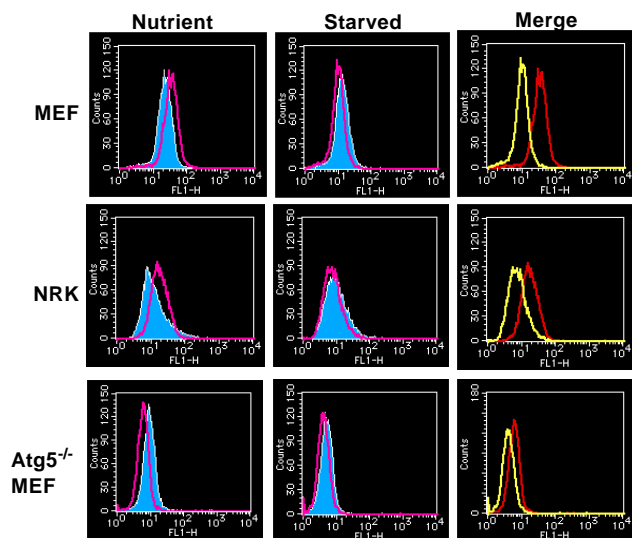
Immunoprecipitation

- 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)

Isotype control Anti-LC3 Nutrient
 Anti-LC3 (M152-3) Anti-LC3 Starved



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

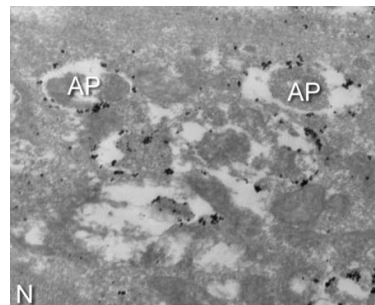
- Shvets, E., and Elazar, Z., *Methods Enzymol.* **452**, 131-141 (2009)
- Shvets, E., *et al.*, *Autophagy* **4**, 621-628 (2008)

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 antigenicity.

- 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 2 times with PBS.
- 7) Resuspend the cells with PBS (5 x 10⁶ 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% NaN₃]. 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 CO₂ 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% glutaraldehyde 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 two 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 two 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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