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POLYCLONAL ANTIBODY

Anti-LC3 pAb

Code No.	Quantity	Form
PM036	100 µL	Purified IgG

BACKGROUND: Macroautophagy mediates the bulk degradation of cytoplasmic components. These components are delivered to lysosomes via autophagosomes. The 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 rabbit serum using protein A agarose. The rabbit was immunized with the recombinant human LC3 [MAP1LC3B (1-120 aa)].

FORMULATION: 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, C) on Western blotting, Immunoprecipitation, Immunohistochemistry, Immunocytochemistry and Flow cytometry. It does not react with GABARAP and GATE-16.

APPLICATIONS:

Western blotting; 1:1,000
Immunoprecipitation; 2 µL/300 µL of cell extract from 1 x 10^7 cells
Immunohistochemistry; 1:1,000-1:2,000
Heat treatment is necessary for paraffin embedded sections.
Microwave oven; twice for 10 minutes each in 10 mM citrate buffer (pH 6.3)
Immunocytochemistry; 1:500-1:1,000
Flow cytometry; 1:200 (final concentration)
Immuno-electron microscopy; Not tested*
*It is reported that this antibody can be used in this application in the reference number 4).

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

INTENDED USE:

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SPECIES CROSS REACTIVITY:

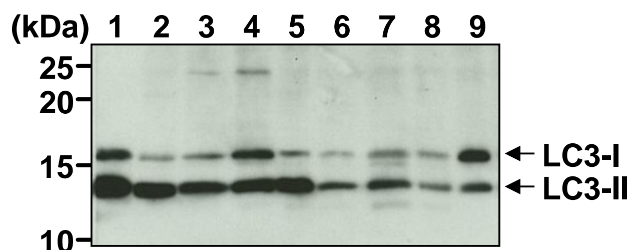
Species	Human	Mouse	Rat	Hamster	Other*
Cells	HeLa, 293T, Raji	NIH/3T3, MEF	PC12, Rat1	CHO	Not tested
Reactivity on WB	+	+	+	+	

*It is reported that this antibody reacts zebrafish tissues in the reference number 1) and 3).

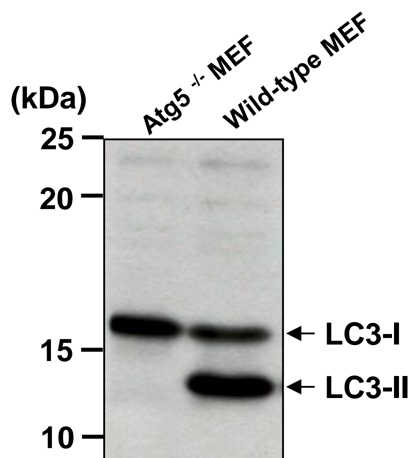
REFERENCES:

1) Festa, B.P., *et al., Nat. Commun*. **9**, 161 (2018) [WB]
2) Wang, Y., *et al*., *Cell* **171**, 331-345.e22 (2017) [IC]
3) Meng, X. H., *et al., Int. J. Biol. Sci*. **13**, 985-995 (2017) [WB]
4) Fujita, N., *et al., Elife* **6**, e23367 (2017) [Immuno-EM]
5) Botbol, Y., *et al*., *Autophagy* **11**, 1864-1877 (2015) [IP]
6) Kaminskyy, V. O., *et al*., *Autophagy* **8**, 1032-1044 (2012) [IC]
7) Kaminskyy, V., *et al*., *Autophagy* **7**, 83-90 (2011) [FCM]
8) Hasui, K., *et al*., *Acta Histochem Cytochem* **44**, 119-131 (2011) [IHC]
9) Tabata, K., *et al*., *Mol. Biol. Cell* **21**, 4162-4172 (2010) [WB]
10) Saitoh, T., *et al*., *Nature* **456**, 264-268 (2008) [WB]
11) Wan, G., *et al*., *J. Biol. Chem*. **283**, 21540-21549 (2008) [WB]
12) Ohne, Y., *et al*., *J. Biol. Chem*. **283**, 31861-31870 (2008) [WB]
13) Kabeya, Y., *et al*., *J. Cell Sci*. **117**, 2805-2812 (2004)
14) Mizushima, N., *et al*., *Mol. Biol. Cell* **15**, 1101-1111 (2004)
15) Mizushima, N., *et al*., *J. Cell Biol*. **152**, 657-667 (2001)
16) Kabeya, Y., *et al*., *EMBO J*. **19**, 5720-5728 (2000)

As this antibody is widely used, many researches have been reported. These references are a part of such reports.



Western blotting analysis of LC3 in positive control (PM036-PN) (1), NIH/3T3 (2), PC12 (3), Rat1 (4), CHO (5), HeLa (6), 293T (7), Raji (8) and mouse brain lysate (9) using PM036.



Western blotting analysis of LC3 in Atg5$^{-/-}$ MEF (left) and wild-type MEF (right) using PM036.

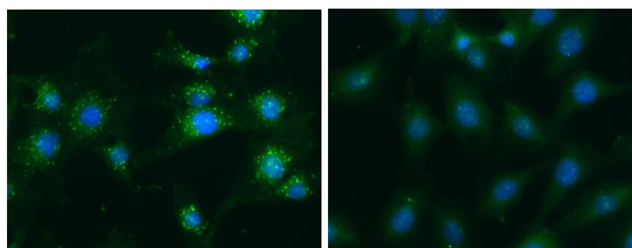
Atg5-/- MEF was kindly provided by Dr. Noboru Mizushima, M.D., Ph.D. (Department of Biochemistry and Molecular Biology, Graduate School and Faculty of Medicine, The University of Tokyo)

PROTOCOLS:

SDS-PAGE & Western Blotting

1) Wash 1 x 10^7 cells 3 times with PBS and suspends them in 1 mL of Laemmli's sample buffer, then sonicate briefly (up to 20 sec.).
2) Boil the samples for 3 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 1% skimmed milk (in PBS, pH 7.2) as suggested in the **APPLICATIONS** for 1 hour at room temperature. (The concentration of antibody will depend on the condition.)
7) Wash the membrane with PBS-T (5 minutes x 3).
8) Incubate the membrane with 1:10,000 Anti-IgG (Rabbit) pAb-HRP (MBL, code no. 458) 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.
11) Remove extra reagent from the membrane by dabbing with paper towel, and seal it in plastic wrap.
12) Expose to an X-ray film in a dark room for 3 minutes.
13) Develop the film as usual. The condition for exposure and development may vary.

(Positive controls for Western blotting; PM036-PN, NIH/3T3, PC12, Rat1, CHO, HeLa, HEK293T, Raji and Mouse brain lysate)

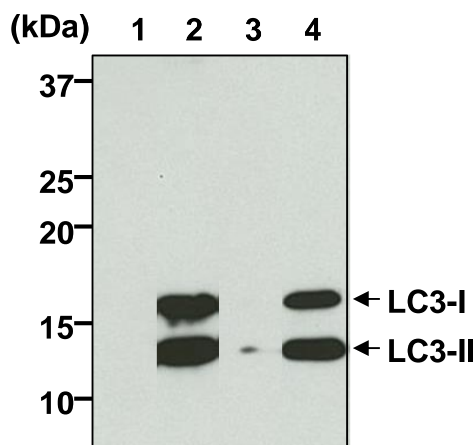


Immunocytochemical detection of LC3 on 4% PFA fixed starved (left) and nutrient NRK cells (right) using PM036.

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/PBS for 10 minutes at room temperature (20~25°C).
5) Wash the slide twice with PBS.
6) Immerse the slides in 100 µg/mL of Digitonin for 10 minutes at room temperature.
7) Wash the slides twice with PBS.
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 slides twice with PBS.
10) Add 200 µL of 1:500 Alexa Fluor® 488 Goat Anti-rabbit IgG (Thermo Fisher Scientific, code no. A11008) diluted with PBS onto the cells. Incubate for 30 minutes at room temperature. Keep out light by aluminum foil.
11) Wash the slides twice with PBS.
12) Counterstain with DAPI for 5 minutes at room temperature.
13) Wash the slides twice with PBS.
14) Wipe excess liquid off the slide but take care not to touch the cells. Never leave the cells to dry.
15) Promptly add mounting medium onto the slide, then put a cover slip on it.

(Positive control for Immunocytochemistry; NRK)



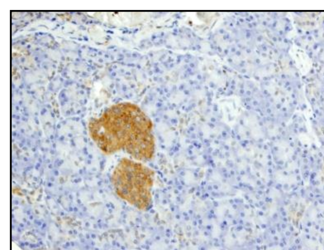
Immunoprecipitation of LC3 from HeLa (1, 2) and NIH/3T3 (3, 4) with Rabbit IgG (1, 3) or PM036 (2, 4). *After immunoprecipitated with the antibody, immunocomplex was resolved on SDS-PAGE and Immunoblotted with M186-3.*

Immunoprecipitation

1) Wash the cells 3 times with PBS and suspend with 10 volume of cold Lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% 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) Mix 20 µL of 50% protein A agarose beads slurry resuspended in 300 µL of IP buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% NP-40] with primary antibody as suggested in the **APPLICATIONS**. Incubate with gentle agitation for 1 hour at 4°C.
4) Wash the beads once with 1 mL of IP buffer.
5) Add 300 µL of cell lysate (prepared sample from step 2) into the tube. Incubate with gentle agitation for 1 hour at 4°C.
6) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
7) Resuspend the agarose with 1 mL of IP buffer.
8) Centrifuge the tube at 2,500 x g for 10 seconds and discard the supernatant.
9) Repeat steps 7)-8) 3 times.
10) Resuspend the beads in 20 µL of Laemmli's sample buffer, boil for 3 minutes, and centrifuge for 5 minutes.
11) Load 10 µL of the sample per lane in a 1-mm-thick SDS-polyacrylamide gel (15% acrylamide) for electrophoresis.
12) 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.
13) To reduce nonspecific binding, soak the membrane in 10% skimmed milk (in PBS, pH 7.2) overnight at 4°C.
14) Wash the membrane with PBS-T [0.05% Tween-20 in PBS] (5 minutes x 3).
15) Incubate the membrane with 1 µg/mL of Anti-LC3 mAb (MBL, code no. M186-3) diluted with 1% skimmed milk (in PBS, pH 7.2) for 1 hour at room temperature. (The concentration of antibody will depend on the conditions.)
16) Wash the membrane with PBS-T (5 minutes x 3).
17) Incubate the membrane with 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.
18) Wash the membrane with PBS-T (5 minutes x 3).
19) Wipe excess buffer on the membrane, then incubate it with appropriate chemiluminescence reagent for 1 minute.
20) Remove extra reagent from the membrane by dabbing with paper towel, and seal it in plastic wrap.
21) Expose to an X-ray film in a dark room for 3 minutes.
22) Develop the film as usual. The condition for exposure and development may vary.

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

Normal human pancreas

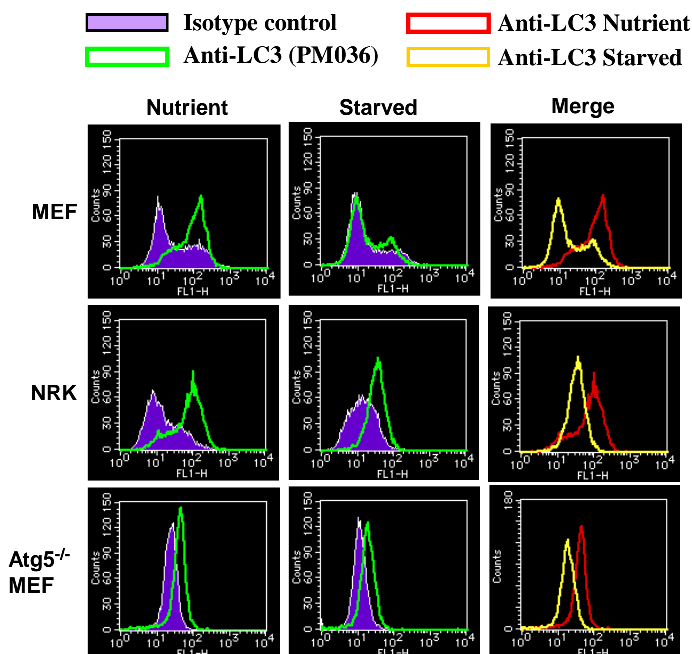


This image is result of immunohistochemical detection analysis using PM036 and is believed to be reliable.

Immunohistochemical staining for paraffin-embedded sections

1) Deparaffinize the sections with Xylene 3 times for 3-5 minutes each.
2) Wash the slides with Ethanol 3 times for 3-5 minutes each.
3) Wash the slides with PBS 3 times for 3-5 minutes each.
4) Heat treatment
   Heat treatment by Microwave:
   Place the slides put on staining basket in 500 mL beaker with 500 mL of 10 mM citrate buffer (pH 6.3). Cover the beaker with plastic wrap, then process the slides twice for 10 minutes each at 500 W with microwave oven. Let the slides cool down in the beaker at room temperature for about 40 minutes.
5) Remove the slides from the citrate buffer and cover each section with 3% H_2O_2 for 10 minutes at room temperature to block endogenous peroxidase activity. Wash 3 times in PBS for 5 minutes each.
6) Remove the slides from PBS, wipe gently around each section and cover tissues with blocking buffer (20 mM HEPES, 1% BSA, 135 mM NaCl) for 5 minutes to block non-specific staining. Do not wash.

7) Tip off the blocking buffer, wipe gently around each section and cover tissues with primary antibody diluted with blocking buffer as suggested in the **APPLICATIONS**.
8) Incubate the sections for 1 hour at room temperature.
9) Wash the slides 3 times in PBS for 5 minutes each.
10) Wipe gently around each section and cover tissues with Histostar™ (Ms + Rb) (MBL, code no. 8460). Incubate for 1 hour at room temperature. Wash as in step 9).
11) Visualize by reacting for 5 minutes with Histostar™ DAB Substrate Solution (MBL, code no. 8469). *DAB is a suspect carcinogen and must be handled with care. Always wear gloves.
12) Wash the slides in water for 5 minutes.
13) Counter stain in hematoxylin for 1 minute, wash the slides 3 times in water for 5 minutes each, and then immerse the slides in PBS for 5 minutes. Dehydrate by immersing in Ethanol 3 times for 3 minutes each, followed by immersing in Xylene 3 times for 3 minutes each.
14) Now ready for mounting.



Flow cytometric analysis of MEF, NRK and Atg5$^{-/-}$MEF cells under starved or nutrient conditions using PM036. *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/PBS for 15 minutes at room temperature (20~25°C). Wash the cells twice with PBS.
5) Permeabilize the cells with 100 µg/mL of Digitonin for 15 minutes at room temperature. Wash the cells twice with PBS.
6) Resuspend the cells with PBS ($5x10^6$ cells/mL).
7) Add 50 µL of the cell suspension into each tube, and centrifuge at 500 x g for 1 minute at room temperature. Remove supernatant by careful aspiration.
8) Add 40 µL of the primary antibody at the concentration as suggested in the **APPLICATIONS** diluted in the washing buffer. Mix well and incubate for 30 minutes at room temperature.
9) 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.
10) Add FITC-conjugated anti-rabbit IgG antibody diluted with the washing buffer. Mix well and incubate for 15 minutes at room temperature.
11) 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.
12) Resuspend the cells with 500 µL of the washing buffer and analyze by a flow cytometer.

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