Store at -20°C

#19848

Autophagy Vesicle Elongation (LC3 Conjugation) Antibody Sampler Kit

1 Kit (6 x 20 µl)

Support: +1-978-867-2388 (U.S.)
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Rev 09/19/16

For Research Use Only. Not For Use In Diagnostic Procedures.

Products Included	Product #	Quantity	Mol. Wt.	Isotype
LC3A/B (D3U4C) XP® Rabbit mAb	12741	20 µl	14, 16 kDa	Rabbit IgG
Atg7 (D12B11) Rabbit mAb	8558	20 µl	78 kDa	Rabbit IgG
Atg4A (D62C10) Rabbit mAb	7613	20 µl	48–60 kDa	Rabbit IgG
Atg4B (D1G2R) Rabbit mAb	13507	20 µl	48 kDa	Rabbit IgG
GABARAP (E1J4E) Rabbit mAb	13733	20 µl	16 kDa	Rabbit IgG
Atg3 Antibody	3415	20 µl	40 kDa	Rabbit
Anti-rabbit IgG, HRP-linked Antibody	7074	100 µl		Goat

See www.cellsignal.com for individual component applications, species cross-reactivity, dilutions and additional application protocols.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

Recommended Antibody Dilutions:

Western blotting	1:1000

For product specific protocols and a complete listing of recommended companion products please see the product web page at www.cellsignal.com

Description: The Autophagy Vesicle Elongation (LC3 Conjugation) Antibody Sampler Kit provides an economical means of detecting target proteins related to autophagy vesicle elongation pathway. The kit contains enough antibody to perform two western blots per primary.

Background: Autophagy is a catabolic process for the autophagosomic-lysosomal degradation of bulk cytoplasmic contents (1,2). Autophagy is generally activated by conditions of nutrient deprivation, but it has also been associated with a number of physiological processes including development, differentiation, neurodegenerative diseases, infection, and cancer (3). Autophagy marker Light Chain 3 (LC3) was originally identified as a subunit of microtubule-associated proteins 1A and 1B (termed MAP1LC3) (4) and subsequently found to contain similarity to the yeast protein Apg8/Aut7/Cvt5 critical for autophagy (5). Three human LC3 isoforms (LC3A, LC3B, and LC3C) undergo post-translational modifications during autophagy (6-8). Cleavage of LC3 at the carboxy terminus immediately following synthesis yields the cytosolic LC3-I form. During autophagy, LC3-I is converted to LC3-II through lipidation by a ubiquitin-like system involving Atg7 and Atg3 that allows for LC3 to become associated with autophagic vesicles (6-9). The presence of LC3 in autophagosomes and the conversion of LC3 to the lower migrating form, LC3-II, have been used as indicators of autophagy (10). Numerous mammalian counterparts to yeast Atg proteins have been described, including three Atg8 proteins (GATE-16, GABARAP, and LC3) and four Atg4 homologs (Atg4A/autophagin-2, Atg4B/autophagin-1, Atg4C/autophagin-3, and Atg4D/autophagin-4) (10-12). The cysteine protease Atg4 is pivotal to autophagosome membrane generation and regulation (13). $GABA_A$ receptor associated protein (GABARAP) is an Atg8 family protein with a key role in autophagy, which was originally discovered as a protein associated with the $GABA_A$ receptor regulating receptor trafficking to the plasma membrane (14). Processing of GABARAP involves cleavage by Atg4 family members (15,16) followed by conjugation by the E1 and E2 like enzymes Atg7 and Atg3 (17,18).

Specificity/Sensitivity: LC3A/B (D3U4C) XP® Rabbit mAb recognizes endogenous levels of total LC3A and LC3B proteins. Atg7 (D12B11) Rabbit mAb recognizes endogenous levels of total Atg7 protein. Atg4B (D1G2R) Rabbit mAb recognizes endogenous levels of total Atg4B protein. Atg4A (D62C10) Rabbit mAb recognizes endogenous levels of total Atg4A protein and recognizes unidentified bands within the molecular weight range of 48-60 kDa, which decrease with silencing of Atg4A expression. GABARAP (E1J4E) Rabbit mAb recognizes endogenous levels of total GABARAP protein, but does not cross-react with other GABARAP family members. Atg3 Antibody detects endogenous levels of total Atg3 protein.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Leu44 of human LC3B protein (conserved in LC3A), a synthetic peptide corresponding to residues near the amino terminus of human Atg7 protein, a synthetic peptide corresponding to residues surrounding Gln100 of human Atg4B protein, a synthetic peptide corresponding to residues near the carboxy terminus of human Atg4A protein, a synthetic peptide corresponding to residues surrounding Arg40 of human GABARAP protein. Polyclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues near the amino terminus of Atg3. Antibodies are purified by protein A and peptide affinity chromatography.

Background References:

(1) Reggiori, F. and Klionsky, D.J. (2002) *Eukaryot Cell* 1, 11-21.

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(3) Levine, B. and Yuan, J. (2005) *J Clin Invest* 115, 2679-88.

(4) Mann, S.S. and Hammarback, J.A. (1994) *J Biol Chem* 269, 11492-7.

(5) Lang, T. et al. (1998) *EMBO J* 17, 3597-607.

(6) He, H. et al. (2003) *J Biol Chem* 278, 29278-87.

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(10) Kabeya, Y. et al. (2004) *J Cell Sci* 117, 2805-12.

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Applications: W—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide **Species Cross-Reactivity: H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse **All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

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Western Immunoblotting Protocol

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween® 20 at 4°C with gentle shaking, overnight.
NOTE: Please refer to primary antibody datasheet or product webpage for recommended primary antibody dilution buffer and recommended antibody dilution.

A. Solutions and Reagents

NOTE: Prepare solutions with reverse osmosis deionized (RODI) or equivalent grade water.

1. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH_2O, mix.
2. **10X Tris Buffered Saline (TBS):** (#12498) To prepare 1 L 1X TBS: add 100 ml 10X to 900 ml dH_2O, mix.
3. **1X SDS Sample Buffer:** Blue Loading Pack (#7722) or Red Loading Pack (#7723) Prepare fresh 3X reducing loading buffer by adding 1/10 volume 30X DTT to 1 volume of 3X SDS loading buffer. Dilute to 1X with dH_2O.
4. **10X Tris-Glycine SDS Running Buffer:** (#4050) To prepare 1 L 1X running buffer: add 100 ml 10X running buffer to 900 ml dH_2O, mix.
5. **10X Tris-Glycine Transfer Buffer:** (#12539) To prepare 1 L 1X transfer buffer: add 100 ml 10X transfer buffer to 200 ml methanol + 700 ml dH_2O, mix.
6. **10X Tris Buffered Saline with Tween® 20 (TBST):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH_2O, mix.
7. **Nonfat Dry Milk:** (#9999)
8. **Blocking Buffer:** 1X TBST with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBST and mix well.
9. **Wash Buffer:** (#9997) 1X TBST
10. **Bovine Serum Albumin (BSA):** (#9998)
11. **Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
12. **Biotinylated Protein Ladder:** (#7727)
13. **Prestained Protein Marker, Broad Range (Premixed Format):** (#7720)
14. **Blotting Membrane and Paper:** (#12369) This protocol has been optimized for nitrocellulose membranes. Pore size 0.2 µm is generally recommended.
15. **Secondary Antibody Conjugated to HRP:** anti-rabbit (#7074); anti-mouse (#7076)
16. **Detection Reagent:** LumiGLO® chemiluminescent reagent and peroxide (#7003) or SignalFire™ ECL Reagent (#6883)

B. Protein Blotting

A general protocol for sample preparation.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl for a 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
6. Microcentrifuge for 5 min.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** Loading of prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights are recommended.
8. Electrotransfer to nitrocellulose membrane (#12369).

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

I. Membrane Blocking

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
3. Wash three times for 5 min each with 15 ml of TBST.

II. Primary Antibody Incubation

1. Incubate membrane and primary antibody (at the appropriate dilution and diluent as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with the species appropriate HRP-conjugated secondary antibody (#7074 or #7076 at 1:2000) and anti-biotin, HRP-linked Antibody (#7075 at 1:1000–1:3000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

D. Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO® #7003, 0.5 ml 20X peroxide, and 9.0 ml purified water) or 10 ml SignalFire™ #6883 (5 ml Reagent A, 5 ml Reagent B) with gentle agitation for 1 min at room temperature.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10 sec exposure should indicate the proper exposure time. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following incubation and declines over the following 2 hr.