Store at -20°C

#70751

Autophagy Vesicle Nucleation Antibody Sampler Kit

1 Kit (8 x 20 µl)

Support: +1-978-867-2388 (U.S.)
www.cellsignal.com/support

Orders: 877-616-2355 (U.S.)
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New 01/16

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

Products Included	Product #	Quantity	Mol. Wt.	Isotype
Atg9A (D4O9D) Rabbit mAb	13509	20 µl	100–110 kDa	Rabbit IgG
Atg14 Antibody	5504	20 µl	65 kDa	Rabbit
Beclin-1 (D40C5) Rabbit mAb	3495	20 µl	60 kDa	Rabbit IgG
Bif-1 Antibody	4467	20 µl	42 kDa	Rabbit
PI3 Kinase Class III (D9A5) Rabbit mAb	4263	20 µl	100 kDa	Rabbit IgG
PIK3R4 Antibody	14580	20 µl	153 kDa	Rabbit
Rubicon (D9F7) Rabbit mAb	8465	20 µl	130 kDa	Rabbit IgG
UVRAG (D2Q1Z) Rabbit mAb	13115	20 µl	90 kDa	Rabbit IgG
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 antibodies.*

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 Nucleation Antibody Sampler Kit provides an economical means of detecting target proteins involved in autophagosome formation and maturation. The kit contains enough antibody to perform two western blot experiments per primary antibody.

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 is also associated with a number of physiological processes including development, differentiation, neurodegeneration, infection and cancer (3). The molecular machinery of autophagy was largely discovered in yeast and is directed by a number of autophagy-related (Atg) genes. These proteins are involved in the formation of autophagosomes, cytoplasmic vacuoles that are delivered to lysosomes for degradation. The PIK3R4/PI3K class III complex interacts with Beclin-1 to play a role during several stages of autophagy. Autophagosome formation is stimulated when Atg14 complexes with PIK3R4, PI3K class III, and Beclin-1. The UVRAG protein competes with Atg14 for Beclin-1 binding, forming a mutually exclusive complex with PIK3R4, PI3K class III, and Beclin-1 that regulates autophagosome maturation. Autophagosome maturation is impaired in the presence of the Beclin-1-binding protein Rubicon (4,5). Co-expression of PIK3R4 is required for PI3K class III activation and regulation by both Beclin-1/UVRAG and nutrient levels (6). Bif-1 directly binds to UVRAG, forming a complex with Beclin-1, resulting in increased PI3-kinase class III/Vps34 activity required for autophagosome maturation (7). Inhibition of GSK-3β, as seen during nutrient deprivation, results in increased expression of Bif-1, and can contribute to autophagic cell death (8). Atg9A is an integral membrane protein that is required for both the initiation and the expansion of the autophagosome (9,10). Recruitment of Atg9A to the autophagosomal membrane is dynamic and transient as Atg9A also cycles between autophagy-related structures known as omegasomes, the trans-Golgi network (TGN), and endosomes, and at no point becomes a stable component of the autophagosomal membrane (9,11). The precise regulation of Atg9A trafficking is not fully clarified, yet it is suggested to involve p38 mitogen-activated protein kinase (MAPK)-binding protein p38IP and the Beclin-1-binding protein Bif-1 (12,13).

Specificity/Sensitivity: Each antibody in the Autophagy Vesicle Nucleation Antibody Sampler Kit detects endogenous levels of its respective target. Rubicon (D9F7) Rabbit mAb detects a band of unknown origin at 55 kDa.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Lys630 of human PI3 kinase class III protein, residues surrounding Thr72 of human Beclin-1 protein, residues surrounding Gly502 of human UVRAG protein, residues surrounding Leu210 of human Rubicon protein, or residues surrounding Gly780 of human Atg9A protein.

Polyclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Gly825 of human PIK3R4 protein, residues surrounding Val215 of human Atg14 protein, or residues surrounding Gly129 of human Bif-1 protein. Polyclonal 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.
(2) Codogno, P. and Meijer, A.J. (2005) *Cell Death Differ* 12 Suppl 2, 1509-18.
(3) Levine, B. and Yuan, J. (2005) *J Clin Invest* 115, 2679-88.
(4) Zhong, Y. et al. (2009) *Nat Cell Biol* 11, 468-76.
(5) Sun, Q. et al. (2008) *Proc Natl Acad Sci USA* 105, 19211-6.
(6) Yan, Y. et al. (2009) *Biochem J* 417, 747-55.
(7) Takahashi, Y. et al. (2007) *Nat Cell Biol* 9, 1142-51.
(8) Yang, J. et al. (2010) *J Cell Sci* 123, 861-70.
(9) Young, A.R. et al. (2006) *J Cell Sci* 119, 3888-900.
(10) Yamada, T. et al. (2005) *J Biol Chem* 280, 18283-90.
(11) Orsi, A. et al. (2012) *Mol Biol Cell* 23, 1860-73.
(12) Webber, J.L. and Tooze, S.A. (2010) *EMBO J* 29, 27-40.
(13) Takahashi, Y. et al. (2011) *Autophagy* 7, 61-73.

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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.