Store at -20°C

Mitophagy Antibody Sampler Kit

#43110

1 Kit
(9 x 20 µl)

Cell Signaling TECHNOLOGY®

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

Products Included	Product #	Quantity	Mol. Wt.	Isotype
SQSTM1/p62 (D5E2) Rabbit mAb	8025	20 µl	62 kDa	Rabbit IgG
NDP52 (D1E4A) Rabbit mAb	60732	20 µl	52, 60 kDa	Rabbit IgG
Optineurin (D2L8S) Rabbit mAb	58981	20 µl	75 kDa	Rabbit IgG
Parkin (Prk8) Mouse mAb	4211	20 µl	50 kDa	Mouse IgG2b
PINK1 (D8G3) Rabbit mAb	6946	20 µl	50, 60 kDa	Rabbit IgG
P-Ubiquitin (S65) (E2J6T) Rabbit mAb	62802	20 µl		Rabbit IgG
BNIP3 (D7U1T) Rabbit mAb	44060	20 µl	22-28, 50-55 kDa	Rabbit IgG
BNIP3L/Nix (D4R4B) Rabbit mAb	12396	20 µl	38, 76	Rabbit IgG
LC3B (D11) XP® Rabbit mAb	3868	20 µl	14, 16	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.

Background: Autophagy is a catabolic process for the autophagosome-lysosomal degradation of bulk cytoplasmic contents (1, 2). Selective autophagy targets the degradation of distinct sets of substrates and organelles (3-5). One of the best studied examples of selective autophagy involves the clearance of damaged mitochondria through a process called mitophagy. Several pathways have been described for various contexts of mitophagy, including the FUNDC1 pathway, the BNIP3 and BNIP3L/Nix pathway, and the PINK1/Parkin pathway. FUNDC1 is a mitochondrial protein that is phosphorylated by the autophagy kinase ULK1 and regulates hypoxia induced mitophagy (6, 7). BNIP3L/Nix and BNIP3 are members of the Bcl-2 family of apoptosis regulators that are expressed on mitochondria, induced by hypoxia, and have have been shown to play a role in mitophagy (8). BNIP3L/Nix is also important in the autophagic maturation of erythroid cells (9). FUNDC1, BNIP3 and BNIP3L/Nix bind to LC3 family members, targeting the mitochondria to the autophagosome.

Non-hypoxic induction of mitophagy can be regulated by the PINK1/Parkin pathway, which plays causative roles in neurodegenerative disease, most notably Parkinson's disease (10, 11). PINK1 is a mitochondrial serine/threonine kinase that is stabilized on the outer mitochondrial membrane of damaged mitochondria. Substrates of PINK1 include the E3 ubiquitin ligase Parkin and ubiquitin itself (12-14). Phosphorylation of Parkin as well as binding to phosphorylated ubiquitin leads to accumulation of ubiquitinated chains on multiple mitochondrial proteins. Ubiquitinated proteins are recognized by selective cargo receptors including SQSTM1/p62, Optineurin, and NDP52 (15-16). Autophagy cargo receptors contain an LC3-interacting region (LIR) required for binding to Atg8/LC3 family members and targeting to the autophagosome (3). The Mitophagy Antibody Sampler Kit provides an economical means of detecting proteins involved in the process of mitophagy. The kit includes enough antibody to perform two western blot experiments with each primary antibody.

Specificity/Sensitivity: Each antibody in the Mitophagy Antibody Sampler kit detects endogenous levels of its target protein. LC3B (D11) XP® Rabbit mAb detects type I and type II forms of LC3B. Weaker reactivity is observed with rodent LC3B. Phospho-Ubiquitin (Ser65) (E2J6T) Rabbit mAb recognizes endogenous levels of Ubiquitin protein only when phosphorylated at Ser65.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with synthetic peptides corresponding to residues surrounding Gly162 of human SQSTM1/p62, Val135 of human NDP52, Pro140 of human PINK1, Leu410 of human Optineurin, Glu128 of human BNIP3L/Nix, peptide sequences corresponding to residues near the amino terminus of human LC3B, the amino terminus of human BNIP3, a recombinant fusion protein with an epitope that maps to the carboxy terminus of human Parkin, and a synthetic phospho-peptide corresponding to residues surrounding Ser65 of human Ubiquitin protein. Antibodies are purified by protein A and peptide affinity chromatography.

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.*

Please visit www.cellsignal.com for validation data and a complete listing of recommended companion products.

Background References:

(1) Reggiori, F. and Klionsky, D.J. (2002) *Eukaryot Cell* 1, 11-21.
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(4) Xu, Z. et al. (2015) *Acta Biochim Biophys Sin (Shanghai)* 47, 571-80.
(5) Mancias, J.D. and Kimmelman, A.C. (2016) *J Mol Biol* 428, 1659-80.
(6) Liu, L. et al. (2012) *Nat Cell Biol* 14, 177-85.
(7) Wu, W. et al. (2014) *EMBO Rep* 15, 566-75.
(8) Sowter, H.M. et al. (2001) *Cancer Res* 61, 6669-73.
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(11) Valente, E.M. et al. (2004) *Science* 304, 1158-60.
(12) Kim, Y. et al. (2008) *Biochem Biophys Res Commun* 377, 975-80.
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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.

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 Detection Pack:** (#7727)
13. **Prestained Protein Marker, Broad Range (Premixed Format):** (#13953)
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 (#13953, 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.

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