Store at -20°C

#24876

Sequestosome Signaling Antibody Sampler Kit

1 Kit (6 x 20 µl)

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

Products Included	Product #	Quantity	Mol. Wt.	Isotype
K63-linkage Specific Polyubiquitin (D7A11) Rabbit mAb	5621	20 µl		Rabbit IgG
KEAP1 (D6B12) Rabbit mAb	8047	20 µl	60–64 kDa	Rabbit IgG
NRF2 (D1Z9C) XP® Rabbit mAb	12721	20 µl	97–100 kDa	Rabbit IgG
SQSTM1/p62 (D5E2) Rabbit mAb	8025	20 µl	62 kDa	Rabbit IgG
TRAF6 (D21G3) Rabbit mAb	8028	20 µl	60 kDa	Rabbit IgG
TrkA (12G8) Rabbit mAb	2510	20 µl	140 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 antibody.*

Recommended Antibody Dilutions:

Western blotting	1:1000

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

Description: The Sequestosome Signaling Antibody Sampler Kit contains reagents to investigate sequestosome signaling within the cell. The kit contains enough antibodies to perform two western blot experiments per primary antibody.

Background: Sequestosome 1 (SQSTM1, p62) is a ubiquitin binding protein involved in cell signaling, oxidative stress, and autophagy (1-4). It was first identified as a protein that binds to the SH2 domain of p56Lck (5) and independently found to interact with PKCζ (6,7). SQSTM1 was subsequently found to interact with ubiquitin, providing a scaffold for several signaling proteins and triggering degradation of proteins through the proteasome or lysosome (8). Interaction between SQSTM1 and TRAF6 leads to the K63-linked polyubiquitination of TRAF6 and subsequent activation of the NF-κB pathway (9). Protein aggregates formed by SQSTM1 can be degraded by the autophagosome (4,10,11). SQSTM1 binds autophagosomal membrane protein LC3/Atg8, bringing SQSTM1-containing protein aggregates to the autophagosome (12). Lysosomal degradation of autophagosomes leads to a decrease in SQSTM1 levels during autophagy; conversely, autophagy inhibitors stabilize SQSTM1 levels. SQSTM1 also interacts with KEAP1, which is a cytoplasmic inhibitor of NRF2, a key transcription factor involved in cellular responses to oxidative stress (3). Under basal conditions, KEAP1 binds and retains NRF2 in the cytoplasm where it can be targeted for ubiquitin-mediated degradation (13). Small amounts of constitutive nuclear NRF2 maintain cellular homeostasis through regulation of basal expression of antioxidant response genes. Following oxidative or electrophilic stress, KEAP1 releases NRF2, thereby allowing the activator to translocate to the nucleus and bind to ARE-containing genes (14). The coordinated action of NRF2 and other transcription factors mediates the response to oxidative stress (15). Thus, accumulation of SQSTM1 can lead to an increase in NRF2 activity (3). KEAP1 also targets the down regulation of NF-κB activity by targeting IKKβ degradation (16). TrkA is a member of Trk receptor tyrosine kinases and is activated by NGF, which stimulates TrkA polyubiquitination (17,18). TrkA regulates proliferation and is important for development and maturation of the nervous system (19). SQSTM1 interaction with TRAF6 controls synthesis of K63 polyubiquititination on TrkA (18, 20). TrkA polyubiquitination is essential for neurotrophin-dependent receptor internalization, cell differentiation, and signaling (18).

Specificity/Sensitivity: Each antibody in the Sequestosome Signaling Antibody Sampler Kit detects endogenous levels of its target protein. K63-linkage Specific Polyubiquitin (D7A11) Rabbit mAb detects polyubiquitin chains formed by Lys63 residue linkage. It does not react with monoubiquitin or polyubiquitin chains formed by linkage to a different lysine residue. TRAF6 (D21G3) Rabbit mAb is not predicted to cross-react with other TRAF family members. TrkA (12G8) Rabbit mAb does not cross-react with TrkB.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Gly162 of human SQSTM1/p62 protein, residues near the amino terminus of human TRAF6 protein, residues surrounding the Lys63 branch of the human diubiquitin chain, residues surrounding Arg220 of human TrkA, residues surrounding Ala275 of human NRF2 protein, and residues near the carboxy terminus of human KEAP1 protein.

Background References:

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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):** (#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.