#12724 Store at -20°C

Ubiquitin E3 Ligase Complex Antibody Sampler Kit

☑ 1 Kit
(8 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
CYLD (D6O5O) Rabbit mAb	12797	20 µl	109 kDa	Rabbit IgG
CUL4A Antibody	2699	20 µl	80, 82 kDa	Rabbit IgG
DDB-1 (D4C8) Rabbit mAb	6998	20 µl	127 kDa	Rabbit IgG
DDB-2 (D4C4) Rabbit mAb	5416	20 µl	43 kDa	Rabbit IgG
RBX1 (D3J5I) Rabbit mAb	11922	20 µl	13 kDa	Rabbit IgG
Skp1 (D3J4N) Rabbit mAb	12248	20 µl	19 kDa	Rabbit IgG
Skp2 (D3G5) XP® Rabbit mAb	2652	20 µl	48 kDa	Rabbit IgG
β-TrCP (D12C8) Rabbit mAb	11984	20 µl	62 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

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

Description: The Ubiquitin E3 Ligase Complex Antibody Sampler Kit provides an economical means to study multiple protein components of ubiquitin E3 ligase complexes. The kit includes enough antibody to perform two western blot experiments with each primary antibody.

Background: Ubiquitin can be covalently linked to many cellular proteins by the ubiquitination process, which targets proteins for degradation by the 26S proteasome. Ubiquitin is first activated by forming a thiolester complex with the activation component E1. The activated ubiquitin is subsequently transferred to the ubiquitin-carrier protein E2, and then from E2 to ubiquitin ligase E3 for final delivery to the ε-NH2 of the target protein lysine residue (1-3). Research studies suggest that activated E2 associates transiently with E3, and the dissociation is a critical step for ubiquitination (4).

S phase kinase-associated protein 1 (Skp1) is a critical scaffold protein of the Skp1/CUL1/F-box (SCF) E3 ubiquitin ligase protein complex. Various F-box proteins (e.g. β-TrCP, Skp2) mediate an interaction with Skp1 via their defining and conserved domain of 40 amino acids and with substrates to be ubiquitinated (5). RING-box protein 1 (RBX1 or ROC1) is another essential component of the SCF complex (6). RBX1 mediates the neddylation of CUL1, which activates SCF E3 ligase by facilitating the ubiquitin transfer from E2 to substrates (7-9). The RING finger domain of RBX1 is required for ubiquitin ligation (10).

Cullin-4 (CUL4) is a member of the cullin family of related ubiquitin ligases (11). The carboxy-terminal domain of CUL4 interacts with Rbx1 and E2 enzyme while the amino-terminal CUL4 domain interacts with BPB domain of UV-damaged DNA binding protein DDB-1 to form a CUL4-DDB1 ubiquitin ligase complex (12). Damaged DNA-Binding Protein (DDB) consists of a 127 kDa subunit (DDB-1) and a 48 kDa subunit (DDB-2) that contribute to the formation of the UV-damaged DNA-binding protein complex (UV-DDB) (13-15). In conjunction with CUL4A and RBX1, the UV-DDB complex forms an E3 ubiquitin ligase that recognizes a broad spectrum of DNA lesions. The complex polyubiquitinates components of the nucleotide excision repair pathway (16-18).

Specificity/Sensitivity: Each antibody in the Ubiquitin E3 Ligase Complex Antibody Sampler Kit recognizes endogenous levels of its respective target protein and does not cross-react with other family members. The Skp2 (D3G5) XP® Rabbit mAb is predicted to cross-react with Skp2a and Skp2B proteins.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with a recombinant protein specific to human CYLD protein, a synthetic peptide corresponding to residues surrounding Gly832 of human DDB-1 protein, to residues surrounding Ala174 of human DDB-2 protein, to the carboxy terminus of human RBX1 protein, to the carboxy terminus of human Skp1 protein, to the amino terminus of human Skp2 protein, and with a recombinant protein specific to human β-TrCP protein. Polyclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Ser12 of human CUL4A protein. Polyclonal antibodies are purified by protein A and peptide affinity chromatography.

Background References:

(1) Ciechanover, A. (1998) *EMBO J* 17, 7151-60.
(2) Hochstrasser, M. (2000) *Nat Cell Biol* 2, E153-7.
(3) Hochstrasser, M. (2000) *Science* 289, 563-4.
(4) Deffenbaugh, A.E. et al. (2003) *Cell* 114, 611-22.
(5) DeSalle, L.M. and Pagano, M. (2001) *FEBS Lett* 490, 179-89.
(6) Zheng, N. et al. (2002) *Nature* 416, 703-9.
(7) Kamura, T. et al. (1999) *Genes Dev* 13, 2928-33.
(8) Morimoto, M. et al. (2003) *Biochem Biophys Res Commun* 301, 392-8.
(9) Pan, Z.Q. et al. (2004) *Oncogene* 23, 1985-97.
(10) Sun, Y. et al. (2001) *Antioxid Redox Signal* 3, 635-50.
(11) Petroski, M.D. and Deshaies, R.J. (2005) *Nat Rev Mol Cell Biol* 6, 9-20.
(12) Lee, J. and Zhou, P. (2007) *Mol Cell* 26, 775-80.
(13) Reardon, J.T. et al. (1993) *J Biol Chem* 268, 21301-8.
(14) Keeney, S. et al. (1993) *J Biol Chem* 268, 21293-300.
(15) Hwang, B.J. and Chu, G. (1993) *Biochemistry* 32, 1657-66.
(16) Chu, G. and Chang, E. (1990) *Proc Natl Acad Sci USA* 87, 3324-7.
(17) Hirschfeld, S. et al. (1990) *Mol Cell Biol* 10, 2041-8.
(18) Payne, A. and Chu, G. (1994) *Mutat Res* 310, 89-102.

Applications Key: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide
Species Cross-Reactivity Key: **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 **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 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.