Rig-I Pathway Antibody Sampler Kit

#8348 Store at -20°C

☑ 1 Kit
(9 x 20 µl)

Cell Signaling TECHNOLOGY®

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rev. 06/16

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

Products Included	Product #	Quantity	Mol. Wt.	Isotype
MDA-5 (D74E4) Rabbit mAb	5321	20 µl	135 kDa	Rabbit IgG
Rig-I (D14G6) Rabbit mAb	3743	20 µl	102 kDa	Rabbit IgG
MAVS Antibody	3993	20 µl	75, 52 kDa	Rabbit IgG
IRF-3 (D6I4C) XP® Rabbit mAb	11904	20 µl	50-55 kDa	Rabbit IgG
TBK1/NAK (D1B4) Rabbit mAb	3504	20 µl	84 kDa	Rabbit IgG
Phospho-TBK1/NAK (Ser172) (D52C2) XP® Rabbit mAb	5483	20 µl	84 kDa	Rabbit IgG
Phospho-IRF-3 (Ser396) (4D4G) Rabbit mAb	4947	20 µl	45-55 kDa	Rabbit IgG
Phospho-IKKε (Ser172) (D1B7) Rabbit mAb	8766	20 µl	80 kDa	Rabbit IgG
IKKε (D20G4) Rabbit mAb	2905	20 µl	80 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.

U.S. Patent No. 5,675,063

Description: The Rig-I Pathway Antibody Sampler Kit provides an economical means to evaluate the activation state and total protein levels of multiple members of the Rig-I pathway including Rig-I, MDA-5, MAVS, IRF-3, TBK1/NAK, and IKKε. The kit includes enough primary antibody to perform two western blot experiments for each primary antibody.

Background: Antiviral innate immunity depends on the combination of parallel pathways triggered by virus detecting proteins in the Toll-like receptor (TLR) family and RNA helicases, such as Rig-I (retinoic acid-inducible gene I) and MDA-5 (melanoma differentiation-associated antigen 5), which promote the transcription of type I interferons (IFN) and antiviral enzymes (1-3). TLRs and helicase proteins contain sites that recognize the molecular patterns of different virus types, including DNA, single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), and glycoproteins. These antiviral proteins are found in different cell compartments; TLRs (i.e. TLR3, TLR7, TLR8, and TLR9) are expressed on endosomal membranes and helicases are localized to the cytoplasm. Rig-I expression is induced by retinoic acid, LPS, IFN, and viral infection (4,5). Both Rig-I and MDA-5 share a DExD/H-box helicase domain that detects viral dsRNA and two amino-terminal caspase recruitment domains (CARD) that are required for triggering downstream signaling (4-7). Rig-I binds both dsRNA and viral ssRNA that contains a 5'-triphosphate end not seen in host RNA (8,9). Though structurally related, Rig-I and MDA-5 detect a distinct set of viruses (10,11). The CARD domain of the helicases, which is sufficient to generate signaling and IFN production, is recruited to the CARD domain of the MAVS/VISA/Cardif/IPS-1 mitochondrial protein, which triggers activation of NF-κB, TBK1/IKKε, and IRF-3/IRF-7 (12-15).

Specificity/Sensitivity: MDA-5 (D74E4) Rabbit mAb, Rig-I (D14G6) Rabbit mAb, MAVS Antibody, IRF-3 (D6I4C) XP® Rabbit mAb, TBK1/NAK (D1B4) Rabbit mAb, and IKKε (D20G4) Rabbit mAb detect endogenous levels of respective total proteins and do not cross-react with other proteins. Bands detected at 52 and 75 kDa by MAVS Antibody correlate with those described by Seth et al. (2005). Phospho-TBK1/NAK (Ser172) (D52C2) XP® Rabbit mAb detects endogenous levels of TBK1/NAK only when phosphorylated at Ser172. This antibody may cross-react with phospho-IKKε. Phospho-IRF-3 (Ser396) (4D4G) Rabbit mAb detects endogenous levels of IRF-3 only when phosphorylated at Ser396. Phospho-IKKε (Ser172) (D1B7) Rabbit mAb recognizes endogenous levels of IKKε protein only when phosphorylated at Ser172. This antibody may cross-react with phospho-TBK1/NAK.

Source/Purification: Polyclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues at the carboxy terminus of human MAVS protein. Polyclonal antibodies are purified by protein A and peptide affinity chromatography. Monoclonal antibodies are produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Arg470 of human MDA-5 protein, Lys652 of human Rig-I protein, Ser645 of human TBK1/NAK protein, Val345 of human IKKε protein, or recombinant human IRF-3 protein. Activation state monoclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Ser172 of human TBK1/NAK protein, Ser396 of human IRF-3 protein, or Ser172 of human IKKε protein.

Background References:

(1) Yoneyama, M. and Fujita, T. (2007) *J Biol Chem* 282, 15315-8.
(2) Meylan, E. and Tschopp, J. (2006) *Mol Cell* 22, 561-9.
(3) Thompson, A.J. and Locarnini, S.A. (2007) *Immunol Cell Biol* 85, 435-45.
(4) Imaizumi, T. et al. (2002) *Biochem Biophys Res Commun* 292, 274-9.
(5) Zhang, X. et al. (2000) *Microb Pathog* 28, 267-78.
(6) Yoneyama, M. et al. (2005) *J Immunol* 175, 2851-8.
(7) Yoneyama, M. et al. (2004) *Nat Immunol* 5, 730-7.
(8) Hornung, V. et al. (2006) *Science* 314, 994-7.
(9) Pichlmair, A. et al. (2006) *Science* 314, 997-1001.
(10) Kato, H. et al. (2006) *Nature* 441, 101-5.
(11) Childs, K. et al. (2007) *Virology* 359, 190-200.
(12) Meylan, E. et al. (2005) *Nature* 437, 1167-72.
(13) Xu, L.G. et al. (2005) *Mol Cell* 19, 727-40.
(14) Kawai, T. et al. (2005) *Nat Immunol* 6, 981-8.
(15) Seth, R.B. et al. (2005) *Cell* 122, 669-82.

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 **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 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.