

Phospho-MLK3 (Thr277/Ser281) Antibody

100 µl
 (10 Western mini-blot)

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This product is for *in vitro* research use only and is not intended for use in humans or animals.

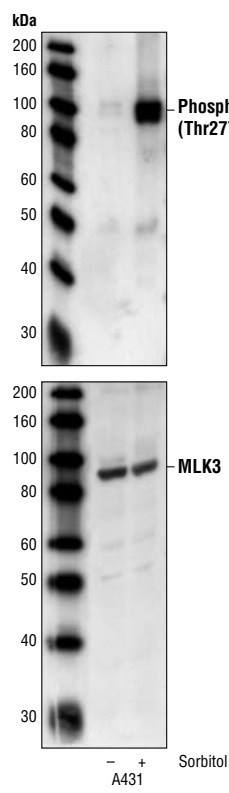
| Applications | Species Cross-Reactivity* | Molecular Wt. | Source |
|--------------|---------------------------|---------------|--------|
| W | H, (M) | 92, 115 kDa | Rabbit |

Background: Mixed lineage kinase 3 (MLK3) is a serine/threonine kinase that has an amino-terminal SH3 domain followed by the kinase domain and two leucine zippers, a cdc42/Rac1 binding (CRIB) domain and several other domains/motifs at the carboxy-terminal region. CRIB triggers the dimerization of MLK3 via its tandem leucine zippers, followed by the intramolecular phosphorylation and subsequent activation of MLK3 (1,2). Autophosphorylation of Thr277 and Ser281 is essential for MLK3 kinase activity (3). Ser281 is also phosphorylated by HPK in an *in vitro* kinase assay (3). MLK3 functions as a MAPKKK of the SAPK/JNK stress pathway by directly phosphorylating SEK1/MKK4 and MKK7, although it is controversial whether MLK3 is involved in p38 stress pathway activation (1,4). MLK3 also functions as an IκB kinase and mediates the activation of the transcriptional factor NF-κB stimulated by CD3/CD28, suggesting a role for MLK3 in immune and inflammatory responses (5).

Specificity/Sensitivity: Phospho-MLK3 (Thr277/Ser281) Antibody detects endogenous levels of MLK3 phosphorylated at Thr277 and Ser281. This antibody does not cross-react with phosphorylated MLK1, MLK2, or other mixed lineage kinases.

Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with a synthetic phospho-peptide (KLH-coupled) corresponding to residues surrounding Thr277/Ser281 of human MLK3. Antibodies are purified by protein A and peptide affinity chromatography.

Background References:
 (1) Teramoto, H. et al. (1996) *J. Biol. Chem.* 271, 27225–27228.
 (2) Leung, I. W. et al. (1998) *J. Biol. Chem.* 273, 32408–32415.
 (3) Leung, I.W. and Lassam, N. (2001) *J. Biol. Chem.* 276, 1961–1967.
 (4) Tibbles, L. A. et al. (1996) *EMBO J.* 15, 7026–7035.
 (5) Hehner, S. P. et al. (2000) *Mol. Cell. Biol.* 20, 2556–2568.



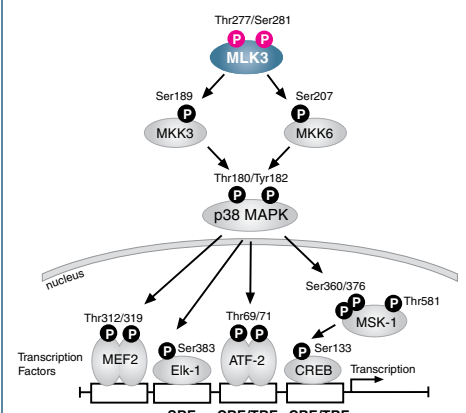
Western blot analysis of extracts from A431 cells, untreated or sorbitol-treated, using Phospho-MLK3 (Thr277/Ser281) Antibody (upper) or MLK3 Antibody #2817 (lower).

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at -20°C. Do not aliquot the antibody.

***Species cross-reactivity is determined by Western blot Recommended Antibody Dilutions:**

Western blotting 1:1000

- Companion Products:**
 Phospho-SEK1/MKK4 (Thr261) Antibody #9151
 Phospho-p38 MAP Kinase (Thr180/Tyr182) Antibody #9211
 p38 MAP Kinase Antibody #9212
 Phospho-p38 MAPK (Thr180/Tyr182) (28B10) Mouse mAb #9216
 PhosphoPlus® SAPK/JNK (Thr183/Tyr185) Antibody Kit #9250
 Phospho-SAPK/JNK (Thr183/Tyr185) Antibody #9251
 SAPK/JNK Antibody #9252
 Phospho-SAPK/JNK (Thr183/Tyr185) (G9) Mouse mAb #9255
 MKK7 Antibody #4172
 Phospho-MKK7(Ser271/Thr275) Antibody #4171
 MLK3 Antibody #2817
 Phototope®-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody #7071
 Anti-rabbit IgG, HRP-linked Antibody #7074
 Prestained Protein Marker, Broad Range (Premixed Format) #7720
 Biotinylated Protein Ladder Detection Pack #7727
 20X LumiGLO® Reagent and 20X Peroxide #7003



IMPORTANT: For Western blots, incubate membrane with diluted antibody in 5% w/v BSA 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry IC—Immunocytochemistry IF—Immunofluorescence
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken X—Xenopus
 Species enclosed in parentheses are predicted to react based on 100% sequence homology.

F—Flow cytometry E—ELISA D—DELFI A®
 Z—zebra fish B—bovine All—all species expected

Western Immunoblotting Protocol (Primary Ab Incubation In BSA)

For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

A Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

- 1X Phosphate Buffered Saline (PBS)
- 1X SDS Sample Buffer:** 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- 10X Tris Buffered Saline (TBS):** To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).
- Nonfat Dry Milk (weight to volume [w/v])
- Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).
- Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
- Bovine Serum Albumin (BSA)
- Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
- Phototope[®]-HRP Western Blot Detection System #7071:** Includes biotinylated protein ladder, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO[®] chemiluminescent reagent and peroxide.
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

B Protein Blotting

A general protocol for sample preparation is described below.

- Treat cells by adding fresh media containing regulator for desired time.
- Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
- Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
- Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
- Microcentrifuge for 5 minutes.
- Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).

NOTE: CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights.

- Electrotransfer to nitrocellulose or PVDF membrane.

C Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm²) of membrane; for different sized membranes, adjust volumes accordingly.

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane with HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.

D Detection of Proteins

- Incubate membrane with 10 ml LumiGLO[®] (0.5 ml 20X LumiGLO[®], 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature.

NOTE: LumiGLO[®] substrate can be further diluted if signal response is too fast.

- Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10-second exposure should indicate the proper exposure time.

NOTE: Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO[®] incubation and declines over the following 2 hours.