#8794 Store at -20°C

SAPK/JNK Kinase Assay Kit (Nonradioactive)

☑ 1 Kit
(40 assays)

Cell Signaling TECHNOLOGY®

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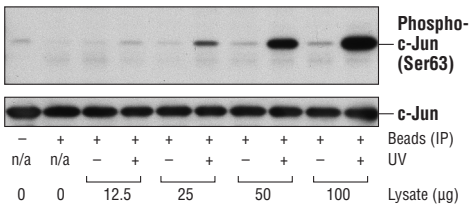
For Research Use Only. Not For Use In Diagnostic Procedures.

Description: The SAPK/JNK Kinase Assay Kit (Nonradioactive) provides all the reagents necessary to measure SAPK/JNK activity in the cell. A phospho-SAPK/JNK Rabbit mAb linked to agarose beads is used to pull down SAPK enzyme from cell extracts. Upon addition of kinase buffer, c-Jun fusion protein and ATP, SAPK phosphorylates the c-Jun substrate. Phospho-c-Jun (Ser63) Antibody can then be used to measure SAPK activity by immunoblotting.

Species Cross Reactivity: H, M

Molecular Weight: 35, 37 kDa



SAPK-induced phosphorylation of c-Jun was measured by quantitative immunoblotting using Phospho-c-Jun (Ser63) Antibody (upper) and c-Jun (L70B11) Mouse mAb #2315 (lower). Lysate titrations were performed by immuoprecipitating phospho-SAPK/JNK from the indicated amounts of HeLa cell extracts, untreated or UV-treated. Phosphorylation of c-Jun at Ser63 is observed in UV-treated lysates.

Kit Components:

Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb (Sepharose® Bead Conjugate): This Cell Signaling Technology antibody is immobilized via covalent binding of primary amino groups to N-hydroxysuccinimide (NHS)-activated Sepharose® beads. Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb (Sepharose® Bead Conjugate) is useful for the immunoprecipitation of SAPK/JNK phosphorylated at Thr183 and Tyr185. The antibody is expected to exhibit the same species cross-reactivity as the unconjugated Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb #4668.

Phospho-c-Jun (Ser63) Antibody: Phospho-c-Jun (Ser63) Antibody recognizes c-Jun only when phosphorylated at Ser63. Polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Ser63 of human c-Jun protein. Antibodies are purified by protein A and peptide affinity chromatography. This antibody is a custom formulation specific to this kit. Polyclonal antibodies are supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at –20°C.

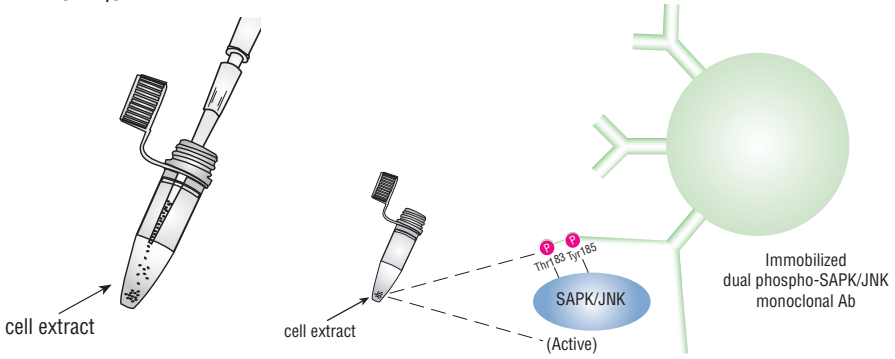
c-Jun Fusion Protein: Isolated from a strain of *E. coli* that carries the coding sequence for human c-Jun residues 1-89 (kindly provided by Dr. J.R. Woodgett). Protein was purified by affinity chromatography.

Products Included	Product #	Kit Quantity
Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb (Sepharose® Bead Conjugate)	4306	400 µl
c-Jun Fusion Protein	6093	40 µg
Kinase Buffer (10X)	9802	15 ml
Cell Lysis Buffer (10X)	9803	15 ml
ATP (10 mM)	9804	50 µl
Anti-rabbit IgG, HRP-linked Antibody	7074	100 µl
Anti-biotin, HRP-linked Antibody	7075	100 µl
20X LumiGLO® Reagent and 20X Peroxide	7003	5 ml each
Biotinylated Protein Ladder	7727	100 µl
Phospho-c-Jun (Ser63) Antibody†	12598	100 µl

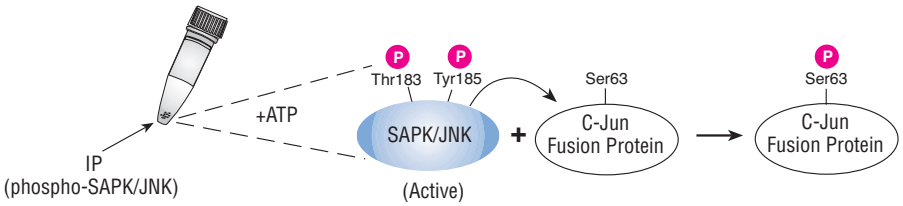
†Antibody #12598 contained in this kit is not available for individual sale.

Kinase Assay Kit Overview

Step 1: Add Immobilized Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb to cell extracts to selectively IP SAPK/JNK.



Step 2: Incubate IP pellets in Kinase Buffer containing c-Jun fusion protein and cold ATP.



Step 3: Detect c-Jun phosphorylation by western blot using Phospho-c-Jun (Ser63) Antibody.

Species Cross-Reactivity Key: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **X**—Xenopus **Z**—zebra fish **B**—bovine **All**—all species expected
Species enclosed in parentheses are predicted to react based on 100% sequence homology.

10X Kinase Buffer: 1X concentration: 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$.

10X Cell Lysis Buffer: 1X concentration: 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml Leupeptin.

10 mM ATP: Adenosine-5' triphosphate (ATP) supplied as a 10 mM solution in sterile, doubly distilled water as a disodium salt.

20X LumiGlo® Reagent and 20X Peroxide: LumiGLO® chemiluminescent substrate is a luminol-based system designed for use with our Phototope-HRP detection assays utilizing peroxidase-labeled antibodies immobilized on membranes. In the presence of hydrogen peroxide, horseradish peroxidase (HRP) converts luminol to an excited intermediate dianion. This dianion emits light on return to its ground state. Light emission is maximal immediately after exposure of the substrate to HRP and continues for 0.5-1 hr. Light can be captured on X-ray film, typically by exposure for a few seconds. Maximum sensitivity can be obtained by longer exposure.

Background: The stress-activated protein kinase/Jun-amino-terminal kinase SAPK/JNK is potently and preferentially activated by a variety of environmental stresses including UV and gamma radiation, ceramides, inflammatory cytokines, and in some instances, growth factors and GPCR agonists (1-6). As with the other MAPKs, the core signaling unit is composed of a MAPKKK, typically MEKK1-MEKK4, or by one of the mixed lineage kinases (MLKs), which phosphorylate and activate MKK4/7. Upon activation, MKKs phosphorylate and activate the SAPK/JNK kinase (2). Stress signals are delivered to this cascade by small GTPases of the Rho family (Rac, Rho, cdc42) (3). Both Rac1 and cdc42 mediate the stimulation of MEKKs and MLKs (3). Alternatively, MKK4/7 can be activated in a GTPase-independent mechanism via stimulation of a germinal center kinase (GCK) family member (4). There are three SAPK/JNK genes each of which undergoes alternative splicing, resulting in numerous isoforms (3). SAPK/JNK, when active as a dimer, can translocate to the nucleus and regulate transcription through its effects on c-Jun, ATF-2, and other transcription factors (3,5).

Background References:

(1) Davis, R.J. (1999) *Biochem Soc Symp* 64, 1-12.

(2) Ichijo, H. (1999) *Oncogene* 18, 6087-93.

(3) Kyriakis, J.M. and Avruch, J. (2001) *Physiol Rev* 81, 807-69.

(4) Kyriakis, J.M. (1999) *J Biol Chem* 274, 5259-62.

(5) Leppä, S. and Bohmann, D. (1999) *Oncogene* 18, 6158-62.

(6) Whitmarsh, A.J. and Davis, R.J. (1998) *Trends Biochem Sci* 23, 481-5.

Nonradioactive IP-Kinase Assay Protocol

A Solutions and Reagents

1. **Note:** Prepare solutions with purified water.
2. **1X Cell Lysis Buffer:** May be stored at 4°C for short-term use (1–2 weeks). **Note:** Supplied 10X Cell Lysis Buffer should be vortexed before being used to make 1X solution.
3. **1X Kinase Buffer:** May be stored at 4°C for short-term use (1–2 weeks).
4. **c-Jun fusion Protein (#6093):** 1 mg/ml. Use 1 µl/assay.
5. **ATP (10 mM) #9804:** 2-(Methylthio)adenosine 5'-triphosphate tetra–sodium salt.
6. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
7. **3X SDS Sample Buffer:** 187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% w/v sodium dodecyl sulfate (SDS), 30% glycerol, 150 mM dithiothreitol (DTT), 0.03% w/v bromophenol blue. For 100 ml, add 2.27 g Tris-HCl, 6 g SDS, 30 ml glycerol, and 30 mg w/v bromophenol blue or bromophenol blue dye, bring to 100 ml with dH_2O. Store at –20°C. Add DTT fresh just before use.
8. **10X Tris-Buffered Saline with Tween®20 (TBS/T) #9997:** 0.2 M Tris base, 1.36 M NaCl, 1.0% Tween®20. To prepare 1 liter, dissolve 24.2 g Tris and 80 g NaCl in dH_2O and adjust pH to 7.6 with HCl. Store at room temperature.
9. **Blocking Buffer:** 1X TBS/T with 5% w/v nonfat dry milk. For 150 ml, dissolve 7.5 g nonfat dry milk in 15 ml 10X TBS/T #9997 and dilute with 135 ml dH_2O. Mix well. Prepare fresh for each experiment.
10. **Wash Buffer:** 1X TBS/T. Store at room temperature.
11. **Primary Antibody Dilution Buffer:** 1X TBS/T with 5% BSA.
12. **Phototope-HRP Western Blot Detection System #7071:** Includes biotinylated protein ladder detection pack (#7727), anti-rabbit IgG, HRP-linked antibody (#7074), anti-biotin, HRP-linked antibody (#7075), 20X LumiGLO® reagent and 20X peroxide (#7003).
13. **LumiGLO® Substrate #7003:** Mix 0.5 ml 20X LumiGLO®, 0.5 ml 20X peroxide, and 9.0 ml dH_2O.

B Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM phenylmethylsulfonyl fluoride (PMSF) to each plate (10 cm^2) and incubate the plate on ice for 5 min.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge at 14,000 x g for 10 min at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C.

C IP with Immobilized Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb

1. **NOTE:** Prior to use, put the tube on ice for 5 min to lower viscosity of buffer. Beads should then be resuspended to a 50% slurry by inversion or gentle vortexing.
2. **For immunoprecipitations with immobilized Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb:** Dilute by adding 10 µl of immobilized Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb bead slurry to 200 µl cell lysate; incubate with gentle rocking overnight at 4°C.

D Kinase Assay

1. Microcentrifuge cell lysate/immobilized Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb at 14,000 x g for 30 sec at 4°C. Wash pellet twice with 500 µl of 1X Cell Lysis Buffer, spinning after each wash. Keep on ice during washes.
2. Wash pellet twice with 500 µl of 1X Kinase Buffer. Keep on ice.
3. Suspend pellet in 50 µl of 1X Kinase Buffer supplemented with 200 µM ATP and appropriate quantity of kinase substrate c-Jun fusion protein (1 µl).
4. Incubate for 30 min at 30°C.
5. Terminate reaction with 25 µl 3X SDS Sample Buffer. Vortex, then microcentrifuge for 30 sec.

E Western Immunoblotting

1. Heat the sample to 95–100°C for 2–5 min.
2. Load 20 µl of sample on SDS-PAGE gel.
3. **Note:** CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein marker (#7727, 10 µl/lane) to estimate molecular weights.
4. Run SDS-PAGE and electrotransfer to nitrocellulose or PVDF membrane. **Note:** Volumes for all the following steps are for 10 cm^2 membrane; for different sized membranes, adjust volumes accordingly.
5. Incubate membrane in 10 ml Blocking Buffer for 1-2 hr at room temperature.
6. Wash three times for 5 min each with 15 ml Wash Buffer.
7. Incubate membrane and antibody (1:1000 dilution) in 10 ml Primary Antibody Dilution Buffer with gentle agitation overnight at 4°C.
8. Wash three times for 5 min each with 15 ml Wash Buffer.
9. Incubate membrane with anti-rabbit IgG, HRP-linked antibody (1:2000) and anti-biotin, HRP-linked antibody (1:1000) to detect biotinylated protein markers in 10 ml of Blocking Buffer with gentle agitation for 1 hr at room temperature.
10. Wash three times for 5 min each with 15 ml Wash Buffer.
11. Incubate membrane with 10 ml LumiGLO® Substrate with gentle agitation for 1 min at room temperature.
12. Drain membrane of excess LumiGLO® Substrate (but 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 LumiGLO® incubation and declines over the following 1-2 hr. LumiGLO® Substrate can be further diluted if signal response is too fast.