#9810 Store at -20°C

SAPK/JNK Kinase Assay Kit (Nonradioactive)

☑ 1 Kit 40 assays

Cell Signaling TECHNOLOGY®

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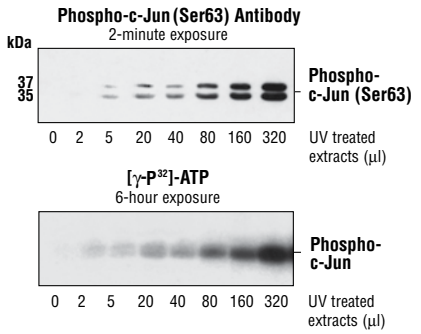
Description: Nonradioactive SAPK/JNK Assay Kit provides all the reagents necessary to measure SAPK/JNK activity in the cell. A c-Jun fusion protein linked to agarose beads is used to pull down SAPK enzyme from cell extracts. Upon addition of kinase buffer and ATP, SAPK phosphorylates the c-Jun substrate.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

Species Cross Reactivity: H, M, R

Molecular Weight: 35, 37 kDa

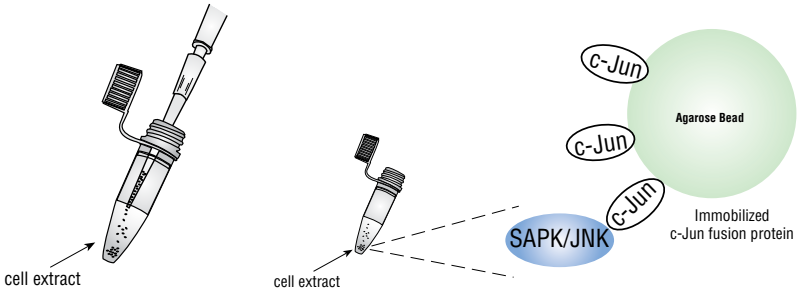


SAPK-induced phosphorylation of c-Jun was measured by quantitative immunoblotting with Phospho-c-Jun (Ser63) Antibody (A) and compared to direct measurement of phosphate incorporation using [gama-32P]-ATP (B). (Note exposure time difference)

Products Included	Product #	Kit Quantity
c-Jun Fusion Protein Beads	9811	1 x 800 µl (40 immunoprecipitations)
Kinase Buffer (10X)	9802	1 x 15 ml
Cell Lysis Buffer (10X)	9803	1 x 15 ml
ATP (10 mM)	9804	1 x 50 µl
Anti-rabbit IgG, HRP-linked Antibody	7074	1 x 100 µl
Anti-biotin, HRP-linked Antibody	7075	1 x 100 µl
20X LumiGLO® Reagent and 20X Peroxide	7003	2 x 5 ml
Biotinylated Protein Ladder Detection Pack	7727	1 x 100 µl
Phospho-c-Jun (S63) Antibody (unique to kit)		100 µl

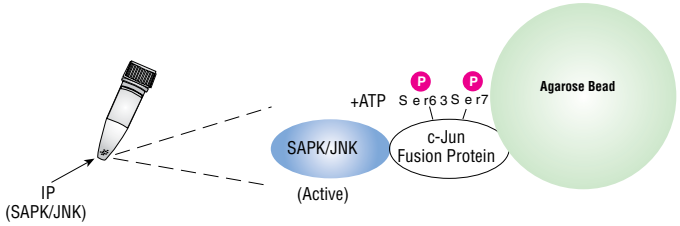
SAPK/JNK Kinase Assay Kit Overview

Step 1: Selective IP of SAPK/JNK using Immobilized c-Jun fusion protein.



a) Add Immobilized c-Jun fusion protein. *b) IP cell extracts using Immobilized c-Jun fusion protein.*

Step 2: Incubate IP pellets in Kinase Buffer containing cold ATP.



Step 3: Analyze c-Jun phosphorylation using phospho-antibodies by western blotting and chemiluminescent detection.

Kit Components:

***Phospho- c-Jun (Ser63) Antibody (rabbit polyclonal IgG, affinity purified):** Phospho- c-Jun (Ser63) antibody detects c-Jun only when catalytically activated by phosphorylation at Ser63. Polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide (KLH coupled) corresponding to residues around Ser63 of human c-Jun. 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.

Purification: Phospho-c-Jun antibodies are purified by protein A and peptide affinity chromatography.

*Note: The antibody provided in this kit is different from phospho- c-Jun (Ser63) II Antibody #9261 currently sold separately. We do not recommend using phospho- c-Jun (Ser63) II Antibody for this assay.

c-Jun (1–89) Fusion Protein Beads: GST fused to the amino-terminus of c-Jun, codons 1–89. Produced from E. coli and provided as a GST-c-Jun fusion bound to glutathione agarose beads. Supplied in 1X lysis buffer containing 50% glycerol. Resuspended beads to a 50% slurry by inversion or ***gentle*** vortexing.

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

Phototope®-HRP Western Detection Kit: The Phototope Western Detection System contains sufficient reagents for the chemiluminescent detection of rabbit antibodies on 10 (10 cm x 10 cm) Western blots. It includes a secondary anti-rabbit antibody conjugated to horseradish peroxidase, anti-biotin antibody conjugated to horseradish peroxidase for the detection of the biotinylated protein ladder (included), LumiGLO® chemiluminescent reagent, and peroxide.

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, by 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 Milli-Q or equivalently 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. **10mM ATP #9804:** 2-(Methylthio)adenosine 5'-triphosphate tetra–sodium salt.
5.* **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
6.* **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, use 2.27 g Tris-HCl, 6g SDS, 30 mL glycerol and 30mg w/v bromophenol blue or bromophenol blue dye. Store at –20°C. Add DTT fresh just before use.
7.* **10X Tris-Buffered Saline with Tween-20 (TBS/T):** 0.2 M Tris base, 1.36 M NaCl, 1.0% Tween-20. To prepare 1 liter, dissolve 24.2 g Tris, 80 g NaCl in dH_2O and adjust pH to 7.6 with HCl. Store at room temperature.
8.* **Blocking Buffer:** 1X TBS/T with 5% w/v nonfat dry milk. For 150 mL, dissolve 7.5g nonfat dry milk in 15 mL 10X TBS/T and 135 mL dH_2O. Mix well. Prepare freshly for each experiment.
9.* **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T). Store at room temperature.
10.* **Primary Antibody Dilution Buffer:** 1X TBS/T with 5% BSA.
11. **Phototope®-HRP Western Blot Detection System #7071:** Includes biotinylated protein marker, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), Anti-biotin, HRP-linked Antibody (#7075), 20X LumiGLO® chemiluminescent reagent and 20X peroxide (#7003).
12. **LumiGLO® Substrate #7003:** 0.5 mL 20X LumiGLO, 0.5 mL 20X peroxide and 9.0 mL Milli-Q water.

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 minutes.
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 minutes 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 c-Jun Fusion protein

1. **For immunoprecipitations with immobilized c-Jun fusion protein:** Dilute by adding 20 µl of immobilized c-Jun fusion protein bead slurry to 200 µL cell lysate; incubate with gentle rocking overnight at 4°C.
2. **Instructions For Use:** Prior to use, put the tube on ice for 5 minutes to lower viscosity of buffer. Then beads should be resuspended to a 50% slurry by inversion or gentle vortexing.

D Kinase Assay

1. Microcentrifuge cell lysate/immobilized c-Jun fusion protein at 14,000 x G for 30 seconds at 4°C. Wash pellet two times 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.
4. Incubate for 30 minutes at 30°C.
5. Terminate reaction with 25 µL 3X SDS Sample Buffer. Vortex, then microcentrifuge for 30 seconds.

E Western Immunoblotting

1. Heat the sample to 95–100°C for 2–5 minutes.
2. Load 20 µl of sample on SDS-PAGE gel.
3. **Note:** CST recommends loading prestained molecular weight markers (#7720, 15 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.
5. **Note:** Volumes for all the following steps are for 10 cm x 10 cm membrane; for different sized membranes, adjust volumes accordingly.
6. Incubate membrane in 10 mL Blocking Buffer for 1-2 hours at room temperature.
7. Wash three times for 5 minutes each with 15 mL Wash Buffer.
8. Incubate membrane and antibody (1:1000 dilution) in 10 mL Primary Antibody Dilution Buffer with gentle agitation overnight at 4°C.
9. Wash three times for 5 minutes each with 15 mL Wash Buffer.
10. 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.
11. Wash three times for 5 minutes each with 15 mL Wash Buffer.
12. Incubate membrane with 10 mL LumiGLO® Substrate with gentle agitation for 1 minute at room temperature.
13. Drain membrane of excess LumiGLO® Substrate (but 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.
14. **Note:** Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO® incubation and declines over the following 1-2 hours. LumiGLO® Substrate can be further diluted if signal response is too fast.

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