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ProductInformation

JNK 1&2 Activity Assay Kit

Product Number **CS0380**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The c-Jun N-terminal kinases (JNKs), also known as stress activated protein kinases (SAPKs), are activated by dual phosphorylation at Thr^{183} and Tyr^{185} in the Thr-Pro-Tyr motif. JNK is potently activated by multiple stresses including inflammatory cytokines, UV-irradiation, osmolarity changes, heat shock and inhibitors of protein synthesis which also regulate the activity of the c-Jun transcription factor.[1] The activated JNKs translocate to the nucleus and function to phosphorylate transcription factors such as c-Jun and ATF2. JNK phosphorylates c-Jun at Ser^{63} and Ser^{73} within its amino terminal activation domain.[2]
The JNK protein kinases are encoded by three genes. The JNK1 and JNK2 gene products are expressed ubiquitously while JNK3 expression is largely restricted to brain, heart and testis.[3] These genes are alternatively spliced to create ten JNK isoforms of 46 kDa and 54 kDa.[4] Regulation of the JNK pathway is extremely complex and is influenced by many Mitogen-activated protein Kinase Kinase Kinases (MKKKs). This diversity of MKKKs allows a wide range of stimuli to activate the Mitogen-activated protein kinase (MAPK) pathway.[2,5] Activation of JNKs in mitogen-stimulated cells appears to be directly mediated by the JNK Kinase SAPK kinase (SEK1) or MAPK Kinase 4 (MKK4), in a signaling pathway involving p21-Activated Kinase (PAK) and MAPK Kinase Kinase (MEKK) in addition to the JNK pathway.[1,5].

The JNK1&2 Activity Assay Kit offers an easy method to assay JNK1&2 activity and to explore new JNK stimuli, inhibitors and activators.[6,7] The kit provides all the reagents required for a straightforward assay for the detection and measurement of JNK activity in cell lysates, tissue homogenates, column fractions or purified kinases. The kit assay is based on immunoprecipitation of the kinase using anti-JNK antibodies, and a detection of the phosphorylation activity of its substrate, ATF2, by immunoblotting, without the need for a secondary antibody. An alternative protocol for radioactive measurement of the JNK activity is also provided.

Reagents

The kit is sufficient for 50 reactions.

- Anti-c-Jun N-terminal Kinase, 0.2 ml, Product Number J 4500
- Detection antibody, 0.5 mg, Monoclonal anti-phospho-ATF2 ($pThr^{69,71}$), Peroxidase conjugate, Product Number A 4728
- Assay Buffer For Kinase Activity, 1 ml, Product Number A 4603 - 75 mM β-glycerophosphate (pH 7.3), 3.75 mM EGTA, 30 mM $MgCl_2$, 4.5 mM DTT, 0.15 mM Na-Vanadate, 0.3 mM ATP
- Wash Buffer 10X, 8 ml, Product Number W 3264 - 500 mM β-glycerophosphate (pH 7.3), 15 mM EGTA, 10 mM $MgCl_2$, 10 mM DTT, 1 mM Na-Vanadate
- EZview™ Red Protein A Affinity Gel, 1 ml, Product Number P 6486
- ATF2, 0.5 mg, Product Number A 2353

Reagents and Equipment required but not provided

- Microfuge centrifuge e.g. Eppendorf® microcentrifuge 5415 Series (Product Number Z60,406-2) or equivalent.
- Dulbecco's Phosphate Buffered Saline (PBS, Product Number D 8537).
- Sample buffer 4x for western blot (preparation instructions are detailed in the Appendix below).
- Materials and equipment required for immunoblot analysis procedure including the ProteoQwest™, chemiluminescent Western blotting kit (Product Code PQ0201) or equivalent.
- Materials and equipment required for the radioactive assay (see below).

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please refer to the Material Safety Data Sheet (MSDS) for information regarding hazards and safe handling practices.

Preparation Instructions

For the reagents preparation and the procedure steps use 17 MOhm water.

1. ATF2 Substrate
   Reconstitute the ATF2 substrate by adding 0.9 ml of 17 MOhm water to the ATF2 substrate bottle, to obtain about 0.6-0.7 mg/ml ATF2. Mix well by pipetting. Aliquot the suspended ATF2 substrate and store at -20°C.
   Note: ATF2 protein is a Maltose binding protein (MBP) fusion protein of 53 kDa with an apparent MW of 60 kDa.
2. Detection antibody
   Reconstitute the detection antibody, Monoclonal anti-phospho-ATF2, by addition of 100 µl of 17 MOhm water to the vial: Mix well by pipetting. Aliquot the suspended antibodies and store at -20°C.
3. Wash buffer 1x
   Dilute the Wash buffer 10x ten fold with 17 MOhm water. Dilute 160 µl of Wash buffer 10X per each sample. For each set of test samples calculate accordingly.

Storage/Stability

The kit is shipped on dry ice and stored at –20 °C. Upon initial thawing, reconstitute the ATF2 substrate and the detection antibodies according to the procedure instructions and freeze them in working aliquots to avoid multiple freeze-thaw cycles.

Procedure

I. Procedure for detection by Immunoblotting

Principle of the assay
JNK is immunoprecipitated from the sample with anti-JNK antibodies and EZview Red Protein A Affinity Gel. The immunoprecipitated JNK phosphorylates the ATF2 and the level of ATF2 phosphorylation is detected by immunoblotting using anti-phospho-ATF2 ($pThr^{69,71}$), Peroxidase conjugate.

The kit can be used for detection of JNK activity in lysates of induced (e.g. UV, anisomycin etc.) versus non-induced cells (as a control), and for the analysis of JNK inhibitors using purified activated JNK.

General comments
1. For each test set two negative control reactions:
   a. Without the addition of the anti-JNK Kinase antibody to detect non-specific protein binding to the agarose.
   b. Without the addition of the ATF2 substrate to determine the existence of endogenous phosphorylated product.
2. All the preparation should be performed on ice unless otherwise stated.

Reaction scheme (example)

	Immunoprecipitation			**Phosphorylation**		**Termination**
	Cell lysate	Anti-JNK	Ezview protein A 50% slurry	Assay buffer 2X	ATF2	Sample buffer 4X
Control 1	250-1000 µl	---------	30 µl	15 µl	15 µl	12 µl
Control 2	250-1000 µl	4 µl	30 µl	15 µl	______	12 µl
Reaction	250-1000 µl	4 µl	30 µl	15 µl	15 µl	12 µl

The procedure described is for one test. Adjust the procedure according to your experiment.

Immunoprecipitation

1. Transfer up to 1 ml of cell lysate/sample (200-500 µg protein) into a microcentrifuge tube. The optimal cell lysate volume is 250 µl.
2. Add 4 µl of anti-JNK Kinase antibody. Mix gently and set the tube on ice while equilibrating the EZview Red Protein A Affinity Gel beads.
3. For equilibrating the EZview Red Protein A Affinity Gel beads:
   a. Carefully mix the gel beads until uniformly suspended. Aliquot 30 µl of the 50 % slurry into a clean 1.5 ml microcentrifuge tube. For beads dispensing, use a wide orifice pipette tip or cut about 1 mm off the tip to enlarge the opening and allow unrestricted flow of the bead suspension.
   b. Wash/equilibrate beads with PBS: Add 750 µl PBS to the tube, vortex and centrifuge in a microcentrifuge for 30 seconds at around 8,000x*g*. Carefully remove the supernatant with a micropipette (or carefully aspirate the supernatant).
   c. Repeat the wash step as indicated above. After removing the supernatant, set the washed bead pellet on ice.

   Note: For tests composed of multiple samples, it is possible to equilibrate together the amount of resin required for all the samples, according to the procedure detailed above. At the last wash, dispense the resin into clean 1.5 ml microcentrifuge tubes according to the number of tests.
4. Briefly centrifuge the tube containing the sample and the antibody (from steps 1-2) for several seconds at around 8,000x*g* to collect all the liquid to the bottom part of the microcentrifuge tube. Carefully transfer all the lysate with a 1 ml micropipette into the tube containing the washed EZview Red Protein A Affinity Gel beads from step 3c.
5. Vortex briefly and incubate with thorough, gentle rocking for 4 hours at 2 - 8°C to allow the antibody-antigen complexes to bind the Protein A on the EZview Red Protein A Affinity Gel beads.
6. Centrifuge the tube in a microcentrifuge for 30 seconds at around 8,000x*g*. Set the tube on ice.
7. Aspirate the supernatant carefully (or remove with a micropipette) and set the tube (containing the bead pellet) on ice.

   Note: Because of the enhanced visibility of the red affinity resin beads, it is easy to see if beads were accidentally removed during the washing steps. If this happens, transfer the washed supernatant back into the tube and repeat the centrifugation step to pellet the resin once again.
8. Wash the bead pellet by adding 400 µl Wash Buffer 1X. Vortex briefly and incubate with a thorough and gentle rocking at 2 - 8°C, for 1 minute.
9. Centrifuge the tube in a microcentrifuge for 30 seconds at around 8,000x*g*. Aspirate the supernatant carefully (or remove with a micropipette) and set the tube with the bead pellet on ice.
10. Perform 2-3 additional washes as indicated in steps 8-9.

ATF2 phosphorylation

1. Add to the bead pellet 15 µl of Assay Buffer and 15 µl of reconstituted ATF2 substrate. Suspend the pellet by gentle pipetting.
2. Incubate for 30 minutes at 30°C.
3. Terminate the reaction with 12µl of SDS sample buffer 4x. Mix well.
4. Boil the sample for 5 minutes, and then spin down for 30 seconds. Save the supernatant.

Immunoblot analysis

1. Load the sample (15-20 µl) on SDS-PAGE gel (10-12%), run the gel and then transfer the proteins to nitrocellulose. Block the nitrocellulose paper using an appropriate blocking solution.
2. To detect the 53 kDa (~60 kDa apparent size) phosphorylated-ATF2, work according to the protocol of the ProteoQwest™, chemiluminescent Western blotting kit (or equivalent). Incubate the membrane with a 1:2000 dilution in blocking buffer of the reconstituted Detection antibody [anti-phopho-ATF2 (pThr^{69,71}), Peroxidase conjugate] . During this incubation step the blot should be gently agitated at room temperature for 2 hours (or alternatively, over-night at 4ºC).
3. Expose the membrane to an x-ray film. An initial 10-second exposure will indicate the need for a different exposure time.

Note: A dot blot procedure could be performed in order to get a sneak preview of the results. Refer to the appendix for procedure.

II. Procedure for radioactive detection

Perform steps 1-10 as described in the procedure for immunoprecipitation section. Continue with the following:

1. Add 1 µl [γ^{32}P]-ATP with a specific activity of 10mCi/ml to 100 µl of Assay Buffer.
2. Suspend the immunoprecipitated pellet by gentle pipetting in 15 µl radioactive Assay buffer and 15 µl reconstituted ATF2 substrate.
3. Incubate the tube for 30 minutes at 30ºC. From this step on, work at room temperature.
4. Terminate the reaction by spotting 25µl of the liquid phase of the sample on 2 cm x 2 cm phosphocellulose P81 squares.
5. Soak the phosphocellulose squares in 0.5% phosphoric acid.
6. Wash the phosphocellulose squares 4 times with 0.5% phosphoric acid. For each wash, agitate gently for 5-6 min.
7. Wash once with ethanol for 1 min.
8. Wash once with acetone for 1 min.
9. Dry the phosphocellulose squares at room temperature or under a heat lamp and count the radioactivity incorporated using Cerenkov mode (i.e. count the emission without scintillation liquid, using tritium channel).

Troubleshooting

Problem	Possible cause	Solution
The signal is very poor or no signal is observed	The amount of activated JNK in the sample is very low	• Add more than 4 µl of anti JNK antibody in step 2 of the immunoprecipitation procedure. • Increase the sample volume - increasing the sample volume up to 1 ml usually has a minor effect on the interaction between the antibody and the activated JNK. • Use a lower dilution of the anti-phospho-ATF2 antibody, Peroxidase conjugate. • Increase the reaction incubation time from 30 min up to 90 min (Step 2 in the ATF2 phosphorylation section or step 3 in the radioactive procedure). • If possible, load on the gel a higher amount of reaction sample.
	There is no activated JNK in the sample	• Prepare a fresh lysate. • Add the appropriate phosphatase inhibitors to the sample (Product Numbers P 2850 and P 5726) or increase their concentration to prevent dephosphorylation of activated JNK. • Add the appropriate protease inhibitors to the sample (Product Number P 8340) or increase their concentration to prevent degradation of activated JNK. • Make sure the extraction buffer is not interfering with the kinase activity. Some extraction buffers that could be used for immunoprecipitation are not suitable for activity assays. We highly recommend using the CelLytic™-M Cell Lysis Reagent (Product Number C 2978) for cell extract preparation. • Verify that the sample is appropriate or that the induction procedure for JNK activation is appropriate. Determine the presence of activated JNK in the sample by immunoblotting of the sample using anti activated JNK antibodies prior to the performance of the immunoprecipitation.
	Incubation time is inadequate	• Prolong the incubation duration of the anti-JNK antibody with the EZview Red Protein A (from several hours to overnight).
	Interfering substance present in sample	• Excessive detergent concentration may interfere with the interaction between the antibody and the activated JNK.
Signal is too strong or is spread out of the lane	There is an excess of ATF2 in the reaction	• Dilute ATF2 substrate with 17 MOhm water before adding it to the reaction tube (dilution from 1:2 to 1:10 is allowable). • Dilute the terminated reaction with more sample buffer or load a smaller volume of reaction sample.
Background is too high or additional bands are visible	Proteins bind non-specifically to Protein A, the resin beads or the microcentrifuge tube	• Pre-clear the sample once or several times by pre-incubation with EZview Red Protein A affinity gel (without the anti-JNK antibody) to remove proteins that may bind non-specifically. • During the final wash (step 10 in the immunoprecipitation procedure), after suspending the resin, transfer the entire sample to a clean microcentrifuge tube before centrifuging the sample.

	Insufficient washes in the immunoprecipitation step	• Increase the number of washes • Prolong the duration of the washes to at least 15 minutes incubation. • Centrifuge at a lower speed to avoid non-specific trapping of lysate proteins during the initial centrifugation of Protein A / antigen complexes.
	Detection antibody is in excess	• Perform a higher dilution of the HRP-conjugated anti-phospho-ATF2 Detection antibody.

Appendix

I. Preparation of Sample buffer 4x

The following procedure is for the preparation of 10 ml Sample buffer 4x.

To 4 ml of 0.5M Tris- HCl pH-6.8 (Trizma base, Product NumberT 1503 titrated to pH-6.8) add:

- 1.2 g SDS (Product Number L 3771)
- 616 mg DTT (Product Number D 0632)
- 10 mg Bromophenol blue (Product Number B 0126)
- 4 g Glycerol (Product Number G 9012).

Complete the volume to 10 ml with water. Mix well.

II. Dot Blot

The Dot blot procedure may be performed in order to get a sneak preview of the results. This procedure does not replace the requirement for a western blot.

1. Cut nitrocellulose membrane into oblong strips of 5 cm x 2 cm (total of 10 cm^2). This is sufficient for 10 samples. Each additional sample (spot) requires an additional membrane of 1 cm^2 area
2. Spot 1µl of the sample that is ready for loading on the gel (from step 4 in the ATF2 phosphorylation detection section) on the nitrocellulose membrane. Let it dry for a few minutes.
3. Rinse briefly with PBS or equivalent.
4. Incubate the membrane in Blocking Solution for 15 minutes.
5. Incubate the membrane with a 1:2000 dilution in blocking buffer of the reconstituted Detection antibody [anti-phospho-ATF2, Peroxidase conjugate]. Incubate with gentle agitation for 20 minutes at room temperature.
6. Wash the membrane five times with PBS containing 0.2% Tween 20 (or equivalent buffer), for 4 minutes each time.
7. Briefly rinse in a wash buffer (with no detergent).
8. Prepare Chemiluminescent Peroxidase Substrate solution, enough to cover the membrane (based on 0.125 ml/cm^2 membrane). Incubate the membrane in the reagent, with gentle agitation, for 1 minute at room temperature.
9. Drain the membrane of excess reagent solution, wrap in plastic wrap and expose to x-ray film. An initial 10 seconds exposure will indicate the need for different exposure times.

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