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JNK 1&2 ELISA
Product Number **CS0100**

Storage Temperature 2-8 °C

ProductInformation

TECHNICAL BULLETIN

Product Description

JNK 1&2 ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) is designed to detect and quantify the level of JNK1 and JNK2 proteins independent of their phosphorylation status. A monoclonal antibody specific for JNK1&2 regardless of phosphorylation state has been coated onto the wells of the multiwell plate provided. JNK 1&2 standard dilutions, control specimens, and unknown samples are pipetted into these wells. During the first incubation, the JNK1&2 antigen binds to the immobilized (capture) antibody. After incubation, the excess reagents are washed away and a rabbit antibody specific for JNK 1&2 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized JNK1&2 protein captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG (Anti-Rabbit IgG-HRP) is added. It binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the excess Anti-Rabbit IgG-HRP, substrate solution is added, which is acted upon by the bound enzyme to produce yellow color. The intensity of this colored product is directly proportional to the concentration of JNK 1&2 present in the original specimen. The optical density measured at 450 nm in a multiwell plate reader is used to calculate the concentration of JNK1&2. The ELISA detects JNK1&2 in human and mouse cell lysates and can be used to normalize the JNK1&2 content of samples when testing for phosphorylated JNK1&2 ($pThr^{183}/pTyr^{185}$) with Sigma Phospho-ELISA (Product No. CS0130).

C-Jun N-terminal Kinase (JNK), also referred to as Stress Activated Protein Kinase (SAPK), is one of the main MAP kinase proteins in mammals. The name JUN comes from the Japanese 'ju-nana,' meaning the number 17. JNK is expressed as ten different isoforms due to differential mRNA splicing. The predominant forms are JNK1 (46 kDa) and JNK2 (54 kDa). JNK is activated by a variety of cellular signals including growth factors, inflammatory cytokines, and environmental stress. The JNK/SAPK signaling pathway involves sequential activation of MAPK kinase kinase (MEKK1), MAPK kinase 4 (MKK4) or MKK7, SAPK/JNK, and c-Jun. Full activation of JNK requires phosphorylation of a threonine and a tyrosine residue in the motif Thr-Pro-Tyr. MKK7 and MKK4 phosphorylate JNK at threonine 183 and tyrosine 185, respectively. The functions of JNK have been facilitated by molecular genetic analysis of JNK signaling in *Drosophila* and knockout mice. These studies demonstrate that the JNK pathway regulates AP-1 transcriptional activity *in vivo* by phosphorylating c-Jun at serines 63 and 73, which increases its transcriptional activation potential. Activation of JNK can elicit either pro-apoptotic or anti-apoptotic signals, depending on cell type. Targeted disruption of JNK genes in primary murine embryonic fibroblasts shows that JNK is required for UV-induced apoptosis, which involves cytochrome c release and the mitochondrial death pathway. A role for JNK in tumor promotion is suggested by the studies in JNK2 knockout mice. Selective inhibition of JNK pathway promotes motor neuron survival and enhances sensitivity of small cell lung cancer cells to cytotoxic compounds. Thus, JNK kinases play an important role in balancing apoptotic and cell survival functions.

Reagents

- **JNK 1&2 Standard, Human, 2 vials, Product No. J 2395 –** lyophilized, full length, human, recombinant JNK2 expressed in *E. coli. Refer to vial label for quantity and reconstitution volume*.
- **Standard Diluent Buffer**, **25 mL**, **Product No. S 3943** - containing BSA and sodium azide as a preservative.
- **Monoclonal Anti-JNK1&2 Antibody-Coated 96 Well Plate, 1 plate, Product No. J 2770**- A plate using break-apart strips coated with monoclonal antibody specific for full length JNK1&2.

- **Anti-JNK1&2 Antibody, 11 mL, Product No. J 2520** – A detection antibody, produced in rabbit, which recognizes JNK1&2 regardless of phosphorylation state. Contains sodium azide and BSA. Ready to use.
- **Anti-Rabbit IgG-HRP Concentrate (100x), 1 vial, 0.125 mL, Product No. R 9402** – contains 3.3 mM thymol and 50% glycerol, . *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No H 8912** –contains 3.3 mM thymol and BSA. Ready to use.
- **Wash Buffer Concentrate 25x, 100 mL, Product No. W 2639** - *See Reagent Preparation for handling, dilution and storage instructions*.
- **Stabilized Chromogen (TMB), 25 mL, Product No. S 3318** – Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm.
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL.
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Graph paper: linear, log-log, or semi-log, as desired.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.

Precautions and Disclaimer

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

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples.
- Thaw completely and mix well prior to analysis.

Denaturing Procedure for Extraction of Proteins from Cells

The antibodies in the JNK1&2 ELISAs require that the protein in cells be denatured in order to achieve a good recognition. A urea-based buffer should be used to prepare cell lysates for JNK1&2 and Phospho-JNK1&2 ($pThr^{183}/pTyr^{185}$) ELISAs. The urea denatures the protein. No sample boiling is necessary for the lysates prepared with this buffer.

Cell Extraction Buffer

6 M urea
0.5% Triton X-100

Protease inhibitor cocktail (Product No. P 2714). Add 250 µL of reconstituted cocktail per 5 mL of Cell Extraction Buffer.
This buffer should be prepared just before lysing the cells.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent). Wash twice with cold PBS.
2. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at –70 ºC and lysed at a later date).
3. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals.
4. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of JNK1&2. For example, 10^8 Jurkat cells grown in RPMI plus 10% FBS can be extracted in 1 mL of Extraction Buffer. Transfer extracts to micro-centrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 ºC.
5. Aliquot the clear lysate to clean microcentrifuge tubes.

Before assay:
Extracted cell lysate samples containing JNK1&2 protein should be diluted with Standard Diluent Buffer *at* least 1:6 to maintain a final urea concentration at less than 1 M.
Under these conditions, 2-10 µL of the clarified cell extract diluted to a volume of 100 µL/well in Standard Diluent Buffer is sufficient for the detection of JNK1&2.

Reagent Preparation

JNK1&2 Standard
JNK 1&2 Standard is prepared from purified full length human recombinant JNK2 expressed in *E. coli*.

1. Reconstitute one vial of Standard with Standard Diluent Buffer according to label directions.
2. Mix gently and wait 10 minutes to ensure complete reconstitution.
3. Label as **20 ng/mL JNK1&2**
4. Prepare serial standard dilutions as follows:

Tube#	Standard Buffer	Standard from tube #:	Final concentration U/mL
1	Reconstitute according to label instructions		20 ng/mL
2	0.25 mL	0.25 mL (1)	10 ng/mL
3	0.25 mL	0.25 mL (2)	5 ng/mL
4	0.25 mL	0.25 mL (3)	2.5 ng/mL
5	0.25 mL	0.25 mL (4)	1.25 ng/mL
6	0.25 mL	0.25 mL (5)	0.625 ng/mL
7	0.25 mL	0.25 mL (6)	0.313 ng/mL
8	0.5 mL	-	0 ng/mL

Mix thoroughly between each dilution.
5. Use within 1 hour of reconstitution.

Anti-Rabbit IgG Horseradish Peroxidase (HRP), 100x Concentrate
Solution contains 50% glycerol and is very viscous. To ensure accurate dilution follow instructions below:
1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Mix: 10 µL IgG-HRP concentrate with 1 mL HRP Diluent (sufficient for one 8-well strip, prepare more if needed)
4. Label as **Anti-Rabbit IgG-HRP Working Solution.**
5. Return the unused Anti-Rabbit IgG-HR concentrate to the refrigerator.

Wash Buffer
1. Equilibrate Wash Buffer Concentrate 25x to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25x with 24 volumes of deionized water
3. Label as **Working Wash Buffer**.
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability

All components of this kit are stable at 2 to 8 ºC for 6 months from the date of purchase.
Any unused reconstituted standard should be discarded or frozen at –70 ºC. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit expiration date. To obtain C of A go to www.sigma-aldrich.com

Procedure

Precautions
- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 ºC).
- Use only the coated 96 well capture plate provided with the kit.
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2 - 8°C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay.
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.

- Read absorbances within 2 hours of assay completion.
- Stabilized Chromogen (TMB) is light sensitive. Avoid prolonged exposure to light. Avoid contact with metal, it may cause color development.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Each wash cycle is repeated four times. Blot as dry as possible after the 4th wash.
- When washing manually, fill wells forcefully with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilutions wells and 2 wells for each sample to be assayed.

Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch.

Summary of JNK1&2 ELISA

1) Incubate 100 µL of Standards and Samples (diluted >1:10) for 2 hours at RT. (Optional: Incubate overnight at 4°C)


aspirate and wash 4x

2) Incubate 100 µL of Detection Antibody for 1 hours at RT.


aspirate and wash 4x

3) Incubate 100 µL of Anti-Rabbit IgG-HRP for 30 minutes at RT.


aspirate and wash 4x

4) Incubate 100 µL of stabilized Chromogen for 30 minutes at RT *(in the dark)*.

5) Add 100 µL of Stop Solution and read at 450nm.

Total Time 4 hours

I. 1st incubation
   a. Add 100 µl Standard Diluent to zero wells.
   b. Add 100 µl standards, samples or controls to the appropriate wells. Note: *Samples lysed in Urea Cell Extraction Buffer must be diluted at least 1:6 in Standard Diluent Buffer to maintain a final urea concentration of less or equal to 1 M. The dilutions should be optimized for each assay.*
   c. Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature. *Alternatively, plate may be incubated overnight at 2 to 8 °C.*
   d. Wash wells 4 times following washing instructions. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

II. 2nd incubation
   a. Add 100 µl Anti-JNK 1&2 detection antibody to all wells (except chromogen blanks).
   b. Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
   c. Wash wells for a total 4 times following washing instructions.
   d. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

III. 3rd incubation
   a. Add 100 µl Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
   b. Cover with Plate Cover and incubate 30 minutes at room temperature.
   c. Wash wells for a total 4 times following washing instructions.
   d. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

IV. Substrate incubation
   a. Add 100 µl of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
   b. Do not cover the plate
   c. Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*
   Note: *The substrate incubation time may vary depending on the plate reader used. If your plate reader does not register optical density (OD) above 2.0, shorten the incubation time to 20-25 minutes.*

V. Stop reaction
   a. Add 100 µl of Stop Solution to each well. This stops the reaction
   b. Tap gently to mix. *The solution will turn yellow.*

VI. Absorbance reading

a. Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.

b. Blank the plate reader against the Chromogen Blank wells (contain Chromogen and Stop Solution).

c. Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution.

Results

The results may be calculated using any immunoassay software package. The four-parameter algorithm provides the best curve fit. If the software program is not readily available, the concentrations of JNK 1&2 may be calculated as follows:

1. Calculate the Average Net OD in nm (average reading of 2 wells) for each standard dilution and samples as follows:
Average Net OD (nm)=
Average Bound OD (nm) – Average Chromogen Blank OD (nm)
2. On graph paper plot the Average Net OD (nm) of standard dilutions against the concentrations (ng/mL) of JNK 1&2 for the standards.
3. Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
4. The JNK 1&2 concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply value(s) obtained for sample(s) by dilution factor to correct for the dilution in step I.b.
6. Samples producing signals higher than the highest standard (20 ng/mL) should be further diluted in *Standard Diluent Buffer* and re-analyzed, multiplying the concentration by the appropriate dilution factor.

Product Profile

Typical Results

The standard curve below is for illustration purposes only and **should not be used** to calculate results in your assay. Run standard curve in each assay. The following data were obtained for the various standards over the range of 0 to 20 ng/mL JNK1&2.

JNK1&2 Standard Curve

Standard ng/mL	OD 450 nm
0	0.146
0.313	0.215.
0.625	0.277
1.25	0.390
2.5	0.606
5.0	1.006
10	1.679
20	2.942

Limitations:

- Do not extrapolate the standard curve beyond the 20 ng/mL standard point.
- The dose response is non-linear in this region and accuracy is compromised.
- Dilute samples >20 ng/mL with *Standard Diluent Buffer;* re-analyze and multiply results by the appropriate dilution factor.
- Other buffers and matrices have not been investigated.
- Although the rate of degradation of native JNK1&2 in the Cell Extraction Buffer described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance Characteristics

Specificity

Recombinant, dually phosphorylated JNK1&2 and non-phosphorylated JNK1&2 were analyzed with Phospho-JNK1&2 (pThr^{183}/pTyr^{185}) ELISA and JNK1&2 ELISA. As shown in Figure 1, JNK1&2 ELISA detects both phosphorylated and non-phosphorylated JNK1&2 proteins, where Phospho-JNK1&2 (pThr^{183}/pTyr^{185}) ELISA only detects JNK1&2 phosphorylated on threonine 183 and tyrosine 185 and does not detect non-phosphorylated JNK 1&2.

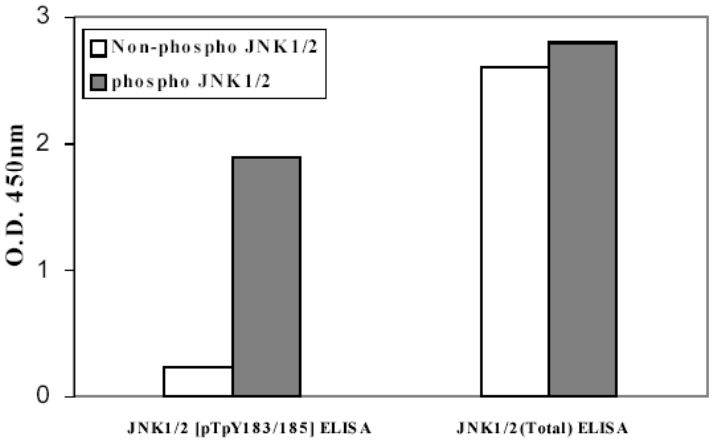

Fig. 1 Specificity of JNK 1&2 ELISA and Phospho-ELISA

JNK1&2 phosphorylation in cells is dependent upon the level of stimulation. Jurkat cells were treated with anisomycin of concentrations 0.001 to 100 µM for 60 minutes, lysed and quantitated in parallel for JNK1&2 (both JNK1&2 and JNK1&2 (pThr^{183}/pTyr^{185}). The results show that while the amount of non-phosphorylated JNK1&2 remains constant among the samples, the levels of JNK phosphorylated at threonine 183 and tyrosine 185 increases with the dosage of anisomycin (Figure 2). The results correlated very well with immunoblot analysis of the same samples (insert).

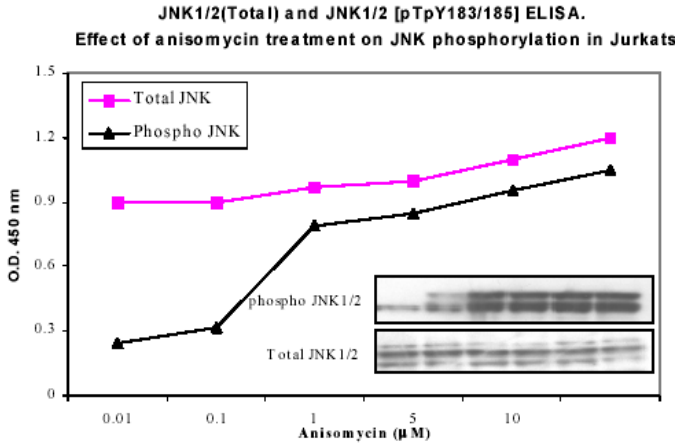

Figure 2 Jurkat cells anisomycin treatment

Sensitivity

Sensitivity of this assay is **<0.15 ng/mL** of human JNK1&2. It was calculated by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. The sensitivity of this ELISA was compared to immunoblotting using known quantities of JNK1&2.

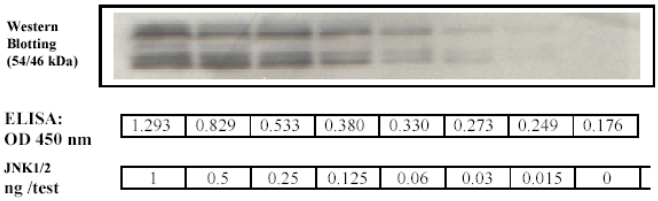

Fig. 3 Detection of JNK 1&2by ELISA vs. immunoblot

The data presented in Figure 3 show that the ELISA is as sensitive as the immunoblotting. The bands shown in the immunoblotting data were developed using mouse monoclonal anti-JNK1&2 and an alkaline phosphatase conjugated anti-mouse IgG followed by chemiluminescent substrate.

Precision

1. Intra-Assay Precision
Samples of known concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	16.67	6.18	1.47
Standard Deviation (SD)	1.34	0.61	0.13
%Coefficient of Variation	8.02	9.80	8.50

2. Inter-Assay Precision
Samples were assayed 42 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (U/mL)	16.22	6.02	1.55
Standard Deviation (SD)	1.38	0.59	0.14
%Coefficient of Variation	8.54	9.80	9.26

Sample Recovery

To evaluate recovery JNK1&2 standard was spiked at 3 different concentrations into 0.5 M and 1 M urea buffer containing 0.5% Triton X-100. The percent recovery ranged from 80-102%.

Parallelism
Natural JNK 1&2from Jurkat cells was extracted in 6 M urea and serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the JNK 1&2 standard curve. Parallelism demonstrated in figure 4 indicated that the standard accurately reflects natural JNK 1&2 content in samples.

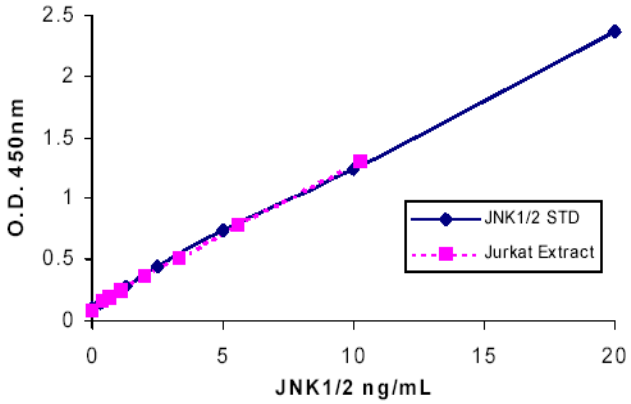

Fig. 4 JNK 1&2ELISA: Parallelism

Linearity of Dilution

Extract Buffer was spiked with recombinant JNK1&2 and serially diluted in Standard Diluent Buffer over the range of the assay and measured for JNK1&2 content.

Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

Cell Lysate			
Dilution	**Measured (ng/mL)**	**Expected (ng/mL)**	**% Expected**
Neat	12	12	100%
1:2	6.2	6.0	103%
1:4	3.1	3.0	105%
1:8	1.4	1.5	96%
1:16	0.7	0.75	93%
1:32	0.32	0.37	81%

References

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