assay designs
SIMPLIFY YOUR SCIENCE®

TiterZyme ®EIA

Total JNK1

Enzyme Immunometric Assay Kit

Catalog No. 900-107

96 Well Kit

Table of Contents

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Description

Assay Designs' Total c-Jun N-terminal protein kinase TiterZyme® Enzyme Immunometric Assay (EIA) kit is a complete kit for the quantitative determintation of pan or total JNK1 in cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to JNK1 immobilized on a microtiter plate to bind the JNK1 in the standards or sample. A recombinant JNK1 Standard is provided in the kit. After a short incubation the excess sample or standard is washed out and a rabbit polyclonal antibody to JNK1 is added. This antibody binds to the JNK1 captured on the plate. The polyclonal antibody detects a non-phosphorylated region of JNK1 and therefore detects both JNK1 and phospho-JNK1. After a short incubation the excess antibody is washed out and goat anti-rabbit IgG conjugated to Horseradish peroxidase is added, which binds to the polyclonal JNK1 antibody. Excess conjugate is washed out and substrate is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of JNK1 in either standards or samples. To measure phospho-JNK1, please refer to Assay Designs' TiterZyme® EIA, Catalog No. 900-106. For further explanation of the principles and practices of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

The c-Jun N-terminal protein kinase (JNK) mitogen-activated protein kinases (MAPKs) are an evolutionarily-conserved family of serine/threonine protein kinases. JNK is also known as stress-activated protein kinase (SAPK). This group of protein kinases act in a signaling system by which cells transduce extracellular stimuli into intracellular responses. Activation of JNK occurs by phosphorylation at Thr^{183} and Tyr^{185} by SEK/MKK4. These stimuli include UV radiation[3], inflammatory cytokines[4], osmotic stress[5] and sheer stress[6]. JNK, when active, can translocate to the nucleus where it regulates transcription through its effects on c-Jun, ATF-2 and other transcription factors [7,8]. The literature contains recent reviews on JNK function[9-11].

Precautions

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

1. Stop Solution 2 is a 1N hydrochloric acid solution. This solution is caustic; care should be taken in use.
2. The activity of the Horseradish peroxidase conjugate is affected by nucleophiles such as azide, cyanide and hydroxylamine.
3. We test this kit's performance with a variety of buffers, however high levels of interfering substances may cause variation in assay results. **For best results samples should be prepared in the buffers recommended and included in this kit**
4. The JNK1 Standard provided, Catalog No. 80-1022, should be handled with care because of the known and unknown effects of JNK1.
5. The JNK1 Standard should be stored at or below -20 °C. Do not repeatedly freeze-thaw.

Materials Supplied

1. **JNK1 Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-1025**
   A plate using break-apart strips coated with a mouse monoclonal antibody specific to JNK1.
2. **Total JNK1 Antibody, 11 mL, Catalog No. 80-1023**
   A yellow solution of rabbit polyclonal antibody to JNK1 & 2.
3. **Assay Buffer 4 Concentrate, 100 mL, Catalog No. 80-0935**
   MOPSO buffered saline containing proteins and detergents.
4. **Total JNK1 Conjugate, 11 mL, Catalog No. 80-1024**
   A blue solution of goat anti-rabbit IgG conjugated to Horseradish peroxidase.
5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
   Tris buffered saline containing detergents.
6. **JNK1 Standard, 0.10 mL, Catalog No. 80-1022**
   A solution of 80,000 pg/mL recombinant JNK1.
7. **TMB Substrate, 11 mL, Catalog No. 80-0350**
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. **Protect from prolonged exposure to light.**
8. **Stop Solution 2, 11 mL, Catalog No. 80-0377**
   A 1N solution of hydrochloric acid in water. Keep tightly capped. Caution: **Caustic.**
9. **Cell Lysis Buffer, 100 mL, Catalog No. 80-0943**
   50 mM β−glycerophosphate, 10 mM HEPES, pH 7.4, 70 mM NaCl, 2 mM EDTA, 1% SDS
10. **Total JNK1 Assay Layout Sheet, 1 each, Catalog No. 30-0180**
11. **Plate Sealer, 3 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the Standard, Assay Buffer 4 Concentrate and Cell Lysis Buffer**, are stable at 4 °C until the kit's expiration date.

The JNK1 Standard **must** be stored at or below -20 °C. The buffers may be stored at room temperature. If the buffers are stored at 4 °C, bring to room temperature and ensure they are in solution prior to use.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Phenylmethylsulfonyl fluoride (PMSF), Sigma #P7626 or equivalent.
3. Protease inhibitor cocktail (PIC), Sigma #P8340 or equivalent.
4. Precision pipets for volumes between 100 µL and 1,000 µL.
5. Repeater pipet for dispensing 100 µL.
6. Disposable beakers for diluting buffer concentrates.
7. Graduated cylinders.
8. A microplate shaker.
9. Adsorbent paper for blotting.
10. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
11. Graph paper for plotting the standard curve.

Sample Handling

Assay Designs' TiterZyme® EIA is compatible with JNK1 samples in a wide range of cell lysates. Samples diluted sufficiently into Assay Buffer 4 plus Inhibitors (see Reagent Preparation, page 5, #2) can be read directly from a standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. It is recommended that all cells be lysed with the provided Cell Lysis Buffer modified by the addition of PMSF and PIC (see Reagent Preparation, page 6, #4) immediately prior to use. Samples lysed in this Cell Lysis Buffer plus Inhibitors must be diluted at least 1:160 with Assay Buffer 4 plus Inhibitors yielding ≤ 6250 cells per mL prior to running the assay. Note that these dilutions are based on the lysis of 1 million cells per mL.

Do not use buffers or components from other kits to prepare samples. **If the end user chooses to use a cell lysis buffer other than the provided Cell Lysis Buffer, it is up to the end user to determine the appropriate dilution of samples and assay validation**. Only standard curves generated in Assay Buffer 4 plus Inhibitors should be used to calculate concentration of JNK1. Samples must be stored frozen at or below -70 °C to avoid loss of bioactive JNK1. Excessive freeze/thaw cycles should be avoided. Prior to assay, frozen samples should be brought to 4 °C slowly and gently mixed.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards must be made up in glass tubes.
4. Pre-rinse the pipet tip with reagent; use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. **Prior to addition of antibody, conjugate and substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. Wash Buffer

Prepare the Wash Buffer by diluting 50 mL of the supplied concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. Assay Buffer 4 plus Inhibitors

Ensure that the Assay Buffer 4 Concentrate is completely in solution prior to use. Prepare the Assay Buffer 4 by diluting 100 mL of the supplied concentrate with 400 mL of deionized water. This can be stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

Immediately prior to use in the assay, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM.

This modified Assay Buffer 4 must be used for all sample and standard dilutions to ensure optimal integrity of JNK1. Fresh Assay Buffer 4 plus Inhibitors must be made for each assay.

3. JNK1 Standards

Allow the 80,000 pg/mL JNK1 standard solution to warm to room temperature. Be sure to briefly centrifuge vial prior to opening to ensure contents are at bottom of vial. Label seven 12x75 mm glass tubes #1 through #7. Pipet 975 µL of Assay Buffer 4 plus Inhibitors into tube #1. Pipet 500 µL of Assay Buffer 4 plus Inhibitors into tubes #2 though #7. Add 25 µL of the 80,000 pg/mL Standard to tube #1. Vortex thoroughly. Add 500 µL of tube #1 to tube #2 and vortex. Add 500 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #7.

The concentration of JNK1 in tubes #1 through #7 will be 2,000, 1,000, 500, 250, 125, 62.5 and 31.25 pg/mL respectively. See JNK1 Assay Layout Sheet for dilution details.

Diluted standards should be used within 20 minutes of preparation.

4. **Cell Lysis Buffer plus Inhibitors**
   Allow to come to room temperature. Ensure buffer is completely in solution prior to use. Immediately prior to use in cell lysis, PMSF and PIC must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM.

   Fresh Cell Lysis Buffer plus Inhibitors must be made each time the cells are lysed.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards, controls and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 100 µL of Assay Buffer 4 plus Inhibitors into the S0 (0 pg/mL standard) wells.
3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Tap the plate gently to mix the contents.
6. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
7. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
8. Pipet 100 µL of yellow Antibody into each well, except the Blank.
9. Seal the plate and incubate at room temperature on a plate shaker for 1 hour at ~500 rpm.
10. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 100 µL of blue Conjugate to each well, except the Blank.
12. Seal the plate and incubate at room temperature on a plate shaker for 30 minutes at ~500 rpm.
13. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 3 more times for a total of **4 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
14. Pipet 100 µL of Substrate Solution into each well.
15. Incubate for 30 minutes at room temperature on a plate shaker at ~500 rpm.
16. Pipet 100 µL Stop Solution 2 to each well. This stops the reaction and the plate should be read immediately.
17. Blank the plate reader against the Blank wells; read the optical density at 450 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all the readings.

Calculation of Results

Several options are available for the calculation of the concentration of JNK1 in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of JNK1 can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Using linear graph paper, plot the Average Net OD for each standard versus JNK1 concentration in each standard. Approximate a straight line through the points. The concentration of JNK1 in the unknowns can be determined by interpolation.

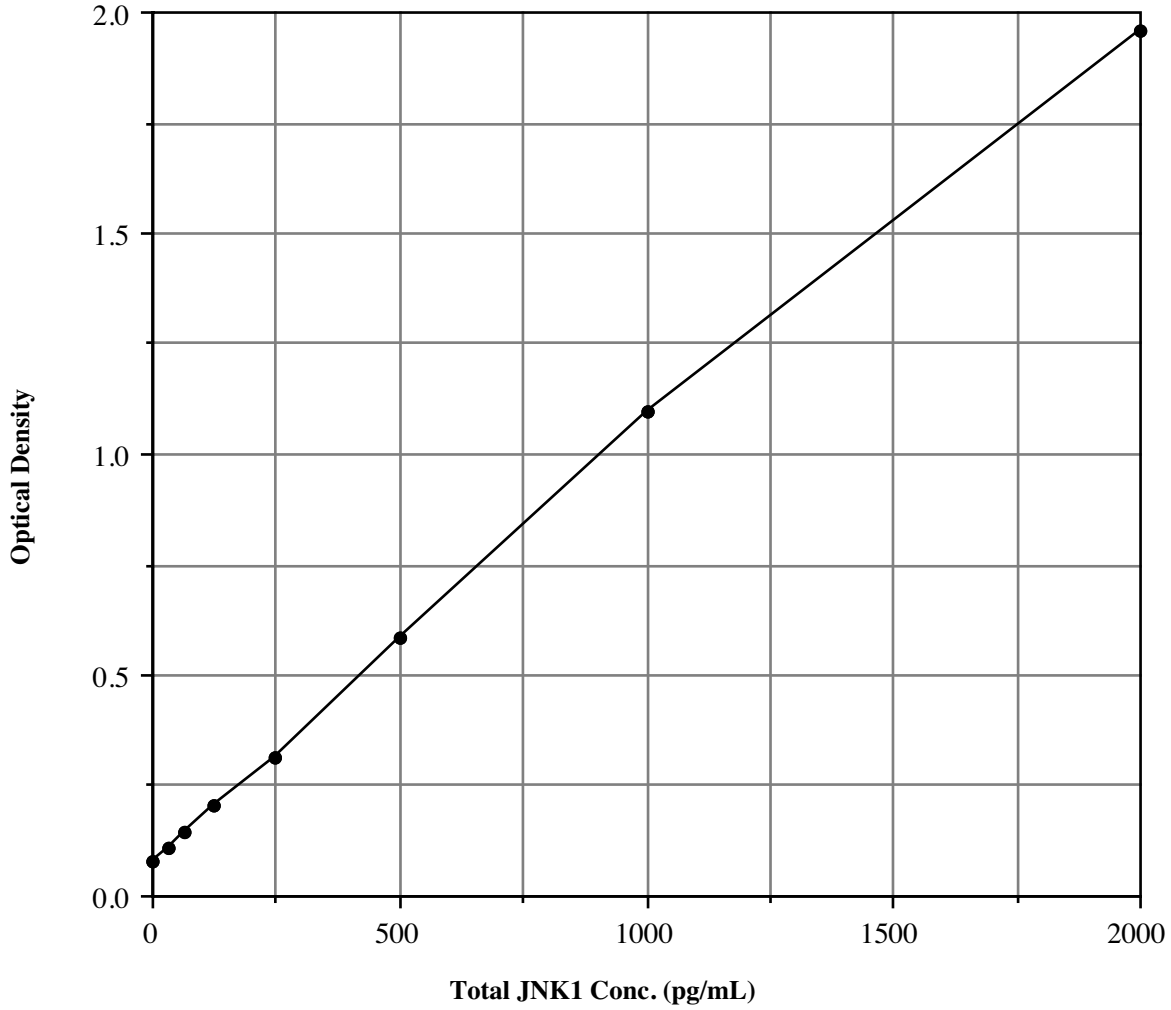
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Average OD	Net OD	**JNK1 (pg/mL)**
Blank	(0.080)		
S0	0.156	0.076	**0**
S1	2.036	1.956	**2,000**
S2	1.177	1.097	**1,000**
S3	0.664	0.584	**500**
S4	0.396	0.316	**250**
S5	0.282	0.202	**125**
S6	0.223	0.143	**62.5**
S7	0.186	0.106	**31.25**
Unknown #1	0.873	0.793	**698**
Unknown #2	0.210	0.130	**57.0**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate JNK1 concentrations; each user must run a standard curve for each assay.



Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[13].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run with 0 pg/mL Standard, and comparing to the average optical density for sixteen (16) wells run with Standard #7. The detection limit was determined as the concentration of JNK1 measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean OD for S0 =	0.089 ± 0.004 (5.0%)
Mean OD for Standard #7 =	0.111 ± 0.007 (6.1%)
Delta Optical Density (31.25 - 0 pg/mL) =	0.111 - 0.089 = 0.022
2 SD's of 0 pg/mL Standard = 2 x 0.004 =	0.008
Sensitivity = $\frac{0.008}{0.022}$ x 31.25 pg/mL =	**11.4 pg/mL**

Linearity

A sample containing 1,535 pg/mL JNK1 was serially diluted 5 times 1:2 in the Assay Buffer 4 plus Inhibitors supplied in the kit and measured in the assay. The data was plotted graphically as actual JNK1 concentration versus measured JNK1 concentration.

The line obtained had a slope of 0.991 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of JNK1 and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of JNK1 in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of JNK1 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	JNK1 (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	59.5	7.8	
Medium	196	3.7	
High	717	4.6	
Low	1,072		7.5
Medium	3,077		9.2
High	7,558		4.3

Cross Reactivities.

The cross reactivities for a number of related compounds were determined by dissolving the cross reactants in the kit's assay buffer at a concentration of 20,000 pg/mL. These samples were then measured in the Total JNK1 assay.

Compound	Cross Reactivity
JNK 1	100%
phospho-JNK1	129%
phospho-JNK2	1.94%
phospho-ERK2	< 0.01%
ERK 2	< 0.01%
phospho-AKT	< 0.01%
AKT	< 0.01%
p38	< 0.01%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

JNK1 concentrations were measured in 1% SDS cell lysates and 1% Triton X-100 cell lysates. JNK1 was spiked into the undiluted samples of these matrices which were then diluted with the kit assay buffer and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
1% SDS / Cell Lysate (1 million cells/mL)	93.3%	≥1:160
1% Triton X-100 / Cell Lysate (1 million cells/ml)	96.8%	≥1:160

WARNING: If the end user chooses to not use the provided 1% SDS cell lysis buffer, it is up the the end user to determine the appropriate dilution of samples and assay validation for their chosen cell lysis buffer.

* See Sample Handling instructions on page 4 for details.

References

1. T. Chard, "An Introduction to Radioimmunoassay & Related Techniques 4th Ed.", (1990) Amsterdam:Elsevier.
2. P. Tijssen, "Practice & Theory of Enzyme Immunoassays", (1985) Amsterdam:Elsevier.
3. M. Seo, et. al., J. Biol. Chem., (2002) [epub ahead of print].
4. A. Kelly, et. al., J. Biol. Chem., (2001) 276:45564-72.
5. C. Rosette and M. Karin, Science, (1996) 274:1194-7.
6. J. Surapisitchat, et. al., Proc. Natl. Acad. Sci. USA, (2001) 98:6476-81.
7. A. Liacini, et. al., Matrix Biol., (2002) 21:251-62.
8. J.A. Recio and G. Merlino, Oncogene, (2002) 21:1000.
9. C.R. Weston, and R.J. Davis, Curr. Opin. Genet. Dev., (2002) 12:14-21.
10. R.K. Barr and M.A. Bogoyevitch, Int. J. Biochem. Cell. Biol., (2001) 33:1047-63.
11. C. Dong, et. al., J. Clin. Immunol., (2001) 21:253-7.
12. E.A. Irving and M. Bamford, J. Cereb. Blood Flow Metab., (2002) 22:631-47.
13. National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.

LIMITED WARRANTY

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

Assay Designs must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if Assay Designs is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

For more details concerning the information within this kit insert, or to order any of Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

Material Safety Data Sheet (MSDS) available on our website or by fax.

Assay Designs, Inc.
5777 Hines Drive
Ann Arbor, MI 48108
U.S.A.

Telephone: **(734) 668-6113**
(800) 833-8651 **(USA & Canada only)**
Fax: **(734) 668-2793**
e-mail: **info@assaydesigns.com**
website: **www.assaydesigns.com**

Simplify Your Science®

Catalog No. 25-0496
Patent Pending

August 10, 2006