[pThr^{183}/Tyr^{185}]Jnk1/2 ELISA kit

Catalog No. ADI-900-106

96 Well Kit

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Description

The [pThr[183]/Tyr[185]]Jnk1/2 ELISA kit is a complete kit for the quantitative determination of pJNK 1/2 in cell lysates. Please read the complete kit insert before performing this assay. The kit uses a monoclonal antibody to JNK immobilized on a microtiter plate to bind the JNK in the standards or sample. A recombinant pJNK1 Standard is provided in the kit. After a short incubation, the excess sample or standard is washed out and a biotinylated monoclonal antibody to pJNK is added. This antibody binds to the pJNK captured on the plate. After a short incubation, the excess antibody is washed out and streptavidin conjugated to Horseradish peroxidase is added, which binds to the biotinylated monoclonal pJNK 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 pJNK in either standards or samples. 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[2], osmotic stress[3] and shear stress[4]. JNK, when active, can translocate to the nucleus where it regulates transcription through its effects on c-Jun, ATF-2 and other transcription factors [5,6]. The literature contains recent reviews on JNK function[7-10].

Precautions

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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 pJNK Standard provided, Catalog No. 80-1119, should be handled with care because of the known and unknown effects of pJNK.
5. The pJNK Standard should be stored at or below -20 °C. Do not repeatedly freeze-thaw.

Materials Supplied

1. **JNK 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 JNK.
2. **pJNK Antibody, 11 mL, Catalog No. 80-1120**
   A yellow solution of biotinylated monoclonal antibody to pJNK.
3. **Assay Buffer 4 Concentrate, 100 mL, Catalog No. 80-0935**
   MOPSO buffered saline containing proteins, detergents and phosphatase inhibitor.
4. **pJNK Conjugate, 11 mL, Catalog No. 80-1121**
   A blue solution of streptavidin conjugated to Horseradish peroxidase.
5. **Wash Buffer Concentrate, 100 mL, Catalog No. 80-1287**
   Tris buffered saline containing detergents.
6. **pJNK Standard, 0.25 mL, Catalog No. 80-1119**
   A solution of 80,000 pg/mL recombinant pJNK1.
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. **RIPA Cell Lysis Buffer 2, 100 mL, Catalog No. 80-1284**
   50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate and 0.1% SDS.
10. **pJNK Assay Layout Sheet, 1 each, Catalog No. 30-0192**
11. **Plate Sealer, 3 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the Standard,** are stable at 4 °C until the kit's expiration date. The Standard **must** be stored at or below -20 °C.

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. Sodium orthovanadate, Sigma #S6508 or equivalent.
5. Sodium pyrophosphate, Sigma #S6422 or equivalent.
6. Precision pipets for volumes between 100 µL and 1,000 µL.
7. Repeater pipet for dispensing 100 µL.
8. Disposable beakers for diluting buffer concentrates.
9. Graduated cylinders.
10. A microplate shaker.
11. Adsorbent paper for blotting.
12. Microplate reader capable of reading at 450 nm, preferably with correction between 570 nm and 590 nm.
13. Graph paper for plotting the standard curve.

Sample Handling

The [pThr^{183}/Tyr^{185}]Jnk1/2 ELISA is compatible with pJnk samples in a wide range of cell lysates. Samples diluted sufficiently into Assay Buffer 4 plus Inhibitors (see Reagent Preparation) 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 samples be lysed with the provided RIPA Cell Lysis Buffer 2 modified by the addition of PMSF and PIC (see Reagent Preparation) immediately prior to use. Samples lysed in this RIPA Cell Lysis Buffer 2 plus Inhibitors must be diluted at least 1:8 with Assay Buffer 4 plus Inhibitors yielding ≤125,000 cells per mL prior to running the assay. Note that this dilution is based on the lysis of 1 million Jurkat cells per mL.

Do not use buffers or components from other kits to prepare samples. If the end user chooses to use another lysis buffer other than the provided RIPA Cell Lysis Buffer 2 or a greater number of cells, 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 the concentration of pJNK. Samples must be stored frozen at or below -70 °C to avoid loss of bioactive pJNK. 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**
   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's 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 PIC 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 pJNK. Fresh Assay Buffer 4 plus Inhibitors must be made for each assay.

3. **pJNK Standards**
   Allow the 80,000 pg/mL pJNK standard solution to warm to room temperature. Label six 12 x 75 mm glass tubes #1 through #6. Pipet 950 µL of Assay Buffer 4 Plus Inhibitors into tube #1. Pipet 500 µL of Assay Buffer 4 plus Inhibitors into tubes #2 through #6. Add 50 µL of the 80,000 pg/mL standard into tube #1 and vortex thoroughly. Add 500 µL of tube #1 to tube #2 and vortex thoroughly. Add 500 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.

   The concentration of pJNK in tubes #1 through #6 will be 4,000, 2,000, 1,000, 500, 250 and 125 pg/mL respectively. See pJNK Assay Layout Sheet for dilution details.

   Diluted standards should be used within 20 minutes of preparation.

4. **RIPA Cell Lysis Buffer 2 plus Inhibitors**
   Allow to come to room temperature. Ensure buffer is completely in solution prior to use. Immediately prior to use in cell lysis, protease inhibitors (PIC and PMSF) as well as phosphatase inhibitors (Sodium orthovanadate and Sodium pyrophosphate) must be added to the buffer. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL PIC or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM. Add Sodium orthovanadate, such as Sigma #S6508, to a final concentration of 2 mM and Sodium pyrophosphate, such as Sigma #S6422, to a final concentration of 20 mM.

   Fresh RIPA Cell Lysis Buffer 2 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 #6 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.
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 pJNK 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 pJNK 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. Plot the Average Net OD for each standard versus pJNK concentration in each standard. Approximate a straight line through the points. The concentration of pJNK in the unknowns can be determined by interpolation.

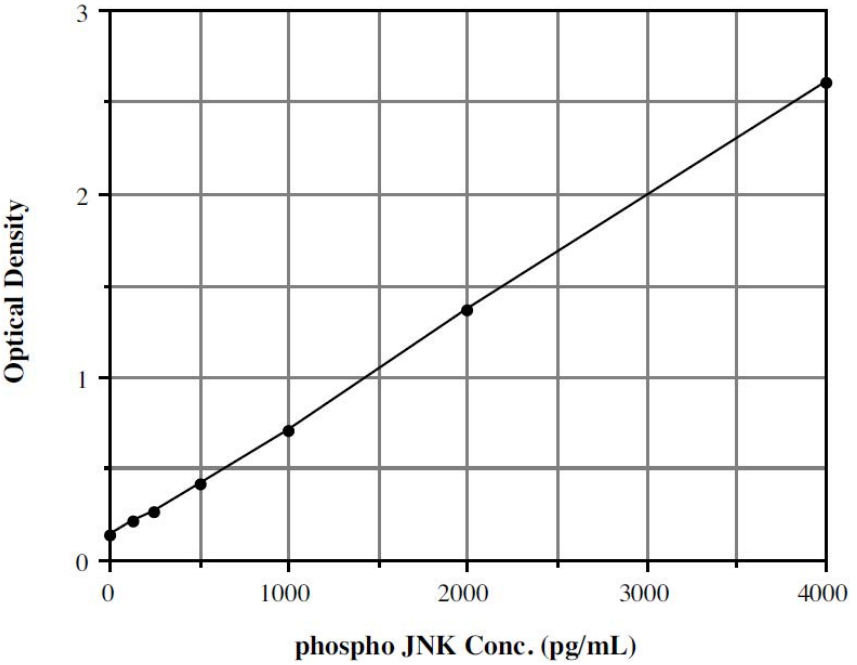
Typical Results

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

Sample	Average OD	Net OD	pJNK (pg/mL)
Blank	(0.052)		
S0	0.189	0.137	**0**
S1	2.660	2.608	**4,000**
S2	1.415	1.363	**2,000**
S3	0.760	0.708	**1,000**
S4	0.472	0.420	**500**
S5	0.316	0.264	**250**
S6	0.264	0.212	**125**
Unknown 1	2.327	2.275	**3,454**
Unknown 2	0.324	0.272	**258**

Typical Standard Curve

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



Units of Measure

Samples measured in the [pThr^{183}/Tyr^{185}]Jnk1/2 ELISA can be expressed in terms of concentration by weight or activity. The Standard Stock concentration is 80,000 pg/mL. To convert this value to Units/mL, the weight concentration is multiplied by the specific activity of the standard. The specific activity of the standard is 179 Units/mg where one Unit of pJNK activity is equal to 1 nmole phosphate incorporated into 2.5 µg/mL ATF2 per minute at 30 °C in a total reaction volume of 50 µL.

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[11].

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 #6. The detection limit was determined as the concentration of pJNK measured at two (2) standard deviations from the 0 pg/mL Standard along the standard curve.

Mean OD for S0 =	0.226 ± 0.020 (8.9%)
Mean OD for Standard #6 =	0.293 ± 0.024 (8.2%)
Delta Optical Density (125 - 0 pg/mL) =	0.066
2 SD's of 0 pg/mL Standard = 2 x 0.015 =	0.040
Sensitivity = $\frac{0.040}{0.066}$ x 125 pg/mL =	**75.8 pg/mL**

Linearity

A sample containing 3,009 pg/mL pJNK was serially diluted 4 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 pJNK concentration versus measured pJNK concentration.

The line obtained had a slope of 0.9941 with a correlation coefficient of 0.9991.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of pJNK 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 pJNK in multiple assays over 3 days (n=8).

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

	pJNK (pg/mL)	Intra-assay % CV	Inter-assay % CV
Low	1,037	8.2	
Medium	1,737	5.2	
High	3,010	4.0	
Low	907		6.5
Medium	1,593		5.6
High	2,963		6.2

Cross Reactivities

The cross reactivities for a number of related compounds were determined by dissolving the cross reactants in the kit assay buffer at a concentration of 40,000 pg/mL. These samples were then measured in the phospho JNK assay.

Compound	Cross Reactivity
phospho-JNK1	100%
phospho-JNK2	98.6%
phospho-p38	0.42%
phospho-ERK2	0.16%
non-phosphorylated ERK2	<0.01%
non-phosphorylated JNK1	<0.01%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation. Phospho-JNK concentrations were measured in RIPA Cell Lysis Buffer 2. pJNK was spiked into the undiluted sample of this matrix which was then diluted with the kit assay buffer and assayed in the kit. The following result was obtained:

Sample	% Recovery*	Recommended Dilution*
RIPA Cell Lysis Buffer 2	102.1%	≥1:8

WARNING: If the end user chooses to not use the provided RIPA Cell Lysis Buffer 2, it is up to 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

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TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

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