DuoSet® IC

Human/Mouse/Rat Phospho-JNK2 (T183/Y185)

Catalog Number DYC2236-2
DYC2236-5
DYC2236E

For the development of sandwich ELISAs to measure JNK2 phosphorylated at T183/Y185 in cell lysates.

This package insert must be read in its entirety before using this product.

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

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PRINCIPLE OF THE ASSAY

This DuoSet® IC ELISA contains the basic components required for the development of sandwich ELISAs to measure JNK2 phosphorylated at T183/Y185 in cell lysates. An immobilized capture antibody specific for JNK2 binds both phosphorylated and unphosphorylated JNK2. After washing away unbound material, a biotinylated detection antibody specific for JNK2 dually phosphorylated at T183 and Y185 is used to detect only phosphorylated protein, utilizing a standard Streptavidin-HRP format.

The detection antibody is an anti-phospho-pan JNK antibody, also recognizing the homologous phosphorylation sites in JNK1 and JNK3. The phospho-JNK2 specificity of this DuoSet IC ELISA is therefore accomplished by the specific capture of JNK2 with the immobilized antibody.

MATERIALS PROVIDED

Store the unopened kit at 2-8° C. Do not use past kit expiration date.

			Vials Provided	
Description	**Part #**	**Storage Conditions**	**Cat. # DYC2236-2**	**Cat. # DYC2236-5**
Phospho-JNK2 (T183/Y185) Capture Antibody	841651	2-8° C	1	2
Phospho-JNK2 (T183/Y185) Detection Antibody	841652	2-8° C	1	2
Phospho-JNK2 (T183/Y185) Standard	841653	2-8° C	3	5
Streptavidin-HRP	890803	2-8° C	1	1

DYC2236-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC2236-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

This kit is also available in an Economy Pack (R&D Systems, Catalog # DYC2236E). Economy Packs contain sufficient materials to run ELISAs on 15 microplates.* Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

*Provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol on page 6.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Sigma #A6279)
- Leupeptin (Tocris # 1167)
- Pepstatin (Tocris # 1190)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma # P7626)
- Sodium Azide (NaN_3) (Sigma # S2002)
- Sodium Fluoride (NaF) (Sigma # 201154)
- Sodium Orthovanadate (Na_3VO_4) (Sigma # S6508), activated
- Sodium Pyrophosphate ($Na_4P_2O_7$) (Sigma # P8010)
- Triton™ X-100 (Sigma # T9284)
- Urea
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates [Costar EIA Plates (Catalog # 2592 or R&D Systems Catalog # DY990) are suggested]
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer.

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 μm filtered.

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalog # WA126).

Block Buffer - 1% BSA* in PBS (pH 7.2-7.4) with 0.05% NaN_3.

IC Diluent #1 - 1% BSA* in PBS, pH 7.2-7.4, 0.2 μm filtered.

IC Diluent #8** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF in PBS, pH 7.2-7.4.

Note: *IC Diluent #8 is also the base diluent for IC Diluent #3, IC Diluent #7, and Lysis Buffer #6. Approximately 50 mL of this diluent is required to run the assay on one plate.*

IC Diluent #3** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 1 M urea in PBS, pH 7.2-7.4.

IC Diluent #7** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea in PBS, pH 7.2-7.4.

Lysis Buffer #6** - 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate, 10 μg/mL Leupeptin, 10 μg/mL Pepstatin, 100 μM PMSF, 3 μg/mL Aprotinin in PBS, pH 7.2-7.4.

Substrate Solution - 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution - 2 N H_2SO_4 (R&D Systems, Catalog # DY994).

*The use of R&D Systems Reagent Diluent Concentrate 2 (R&D Systems, Catalog # DY995) or Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2-8° C.

**Sample Diluent Concentrate 1 (5X) (R&D Systems, Catalog # DYC001), prepared as described in the DYC001 insert.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Phospho-JNK2 (T183/Y185) Capture Antibody (Part 841651) - Each vial contains 360 μg/mL of mouse anti-human JNK2 antibody when reconstituted with 200 μL of PBS. After reconstitution, store at 2-8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Phospho-JNK2 (T183/Y185) Detection Antibody (Part 841652) - Each vial contains 9 μg/mL of biotinylated rabbit anti-human phospho-JNK2 (T183/Y185) antibody when reconstituted with 1.0 mL of IC Diluent #1. After reconstitution, store at 2-8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Phospho-JNK2 (T183/Y185) Standard (Part 841653) - Each vial contains 320 ng/mL of recombinant human phospho-JNK2 (T183/Y185) when reconstituted with 500 μL of IC Diluent #7. **Use within one hour after reconstitution. A fresh standard should be used for each assay.** Immediately before use, an initial 6-fold dilution should be made in IC Diluent #8. Additional dilutions should be made in IC Diluent #3. A seven point standard curve using 2-fold dilutions and a high standard of 8000 pg/mL is recommended.

Streptavidin-HRP (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Store at 2-8° C. **DO NOT FREEZE**.

*Provided this is within the expiration date of the kit.

PREPARATION OF SAMPLES

Cell Lysates - Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer #6 and allow samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70° C. Before use, centrifuge samples at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Sample protein concentration may be quantified using a total protein assay. For assaying, dilute lysates 6-fold with IC Diluent #8 and make further serial dilutions in IC Diluent #3.

Note: *The final concentration of urea in all samples should be 1 M prior to adding to the plate.*

PRECAUTION

The Stop Solution suggested for use with this kit is an acidic solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

TECHNICAL HINTS AND LIMITATIONS

- This DuoSet IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is important that the diluents selected for reconstitution and for dilution of the standard reflect the environment of the samples being measured. The diluent suggested in this protocol should be suitable for most cell lysates.
- The type of enzyme and substrate and the concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8° C or be prepared fresh daily.

GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 2.0 μg/mL in PBS, without carrier protein. Immediately coat a 96 well microplate with 100 μL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of 3 washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 μL of Block Buffer to each well. Incubate at room temperature for 1-2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 μL of sample or standards in IC Diluent #3 per well. Use IC Diluent #3 as the zero standard. Cover with a plate sealer and incubate 2 hours at room temperature.

   Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 8000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Dilute the Detection Antibody to a working concentration of 250 ng/mL in IC Diluent #1 before use. Add 100 μL of the diluted Detection Antibody to each well. Cover with a new plate sealer and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using IC Diluent #1. Add 100 μL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
7. Add 100 μL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

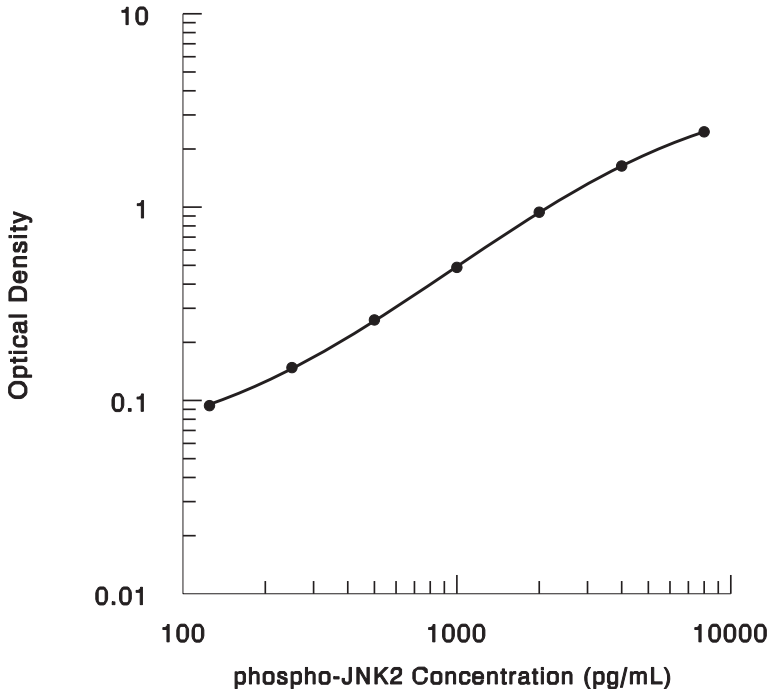
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample then subtract the average zero standard optical density. Results may be normalized to total protein or cell number.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the phospho-JNK2 (T183/Y185) concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

TYPICAL DATA

A standard curve should be generated for each set of samples assayed. The graph below represents typical data generated when using this Human/Mouse/Rat Phospho-JNK2 (T183/Y185) DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



CALIBRATION

The Human/Mouse/Rat Phospho-JNK2 (T183/Y185) DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-JNK2 (T183/Y185) produced at R&D Systems. Samples containing natural phospho-JNK2 (T183/Y185) showed linear dilution parallel to the standard curve obtained using the phospho-JNK2 (T183/Y185) Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the relative concentration of phospho-JNK2 (T183/Y185) in natural samples.

SPECIFICITY

The Human/Mouse/Rat Phospho-JNK2 (T183/Y185) DuoSet IC ELISA specifically recognizes JNK2 dually phosphorylated at T183 and Y185. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody supplied in the kit.

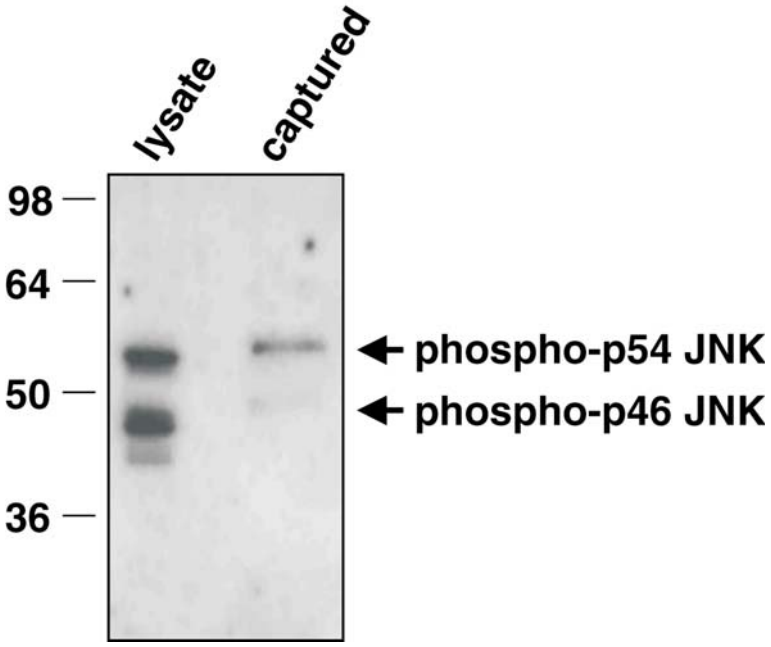


Figure 1: Lysates prepared from HepG2 human hepatocellular carcinoma cells treated with 10 ng/mL of IL-1β (R&D Systems, Catalog # 201-LB) were incubated in wells coated with Phospho-JNK2 Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to a PVDF membrane and immunoblotted with Phospho-JNK2 Detection Antibody. Two major bands corresponding to the phosphorylated p54 and p46 splice variants of JNK1, JNK2 and JNK3 were detected by Western blot in the lysate sample. Because p54 is the predominant isoform of JNK2, this band is more visible than the p46 band in the material captured by the JNK2-specific antibody provided in this DuoSet IC ELISA.

To further determine specificity, recombinant human (rh) JNK1 phosphorylated at T183 and Y185, and rhJNK3 phosphorylated at T221 and Y223 were assayed at 50 ng/mL and did not cross-react or interfere in the assay. Recombinant human JNK2 was assayed at 50 ng/mL and did not cross-react or interfere. Recombinant human ERK2 and rhp38α were assayed at 200 ng/mL and did not cross-react or interfere in the assay.

QUANTIFICATION

Amounts of human phosphorylated JNK2, as quantified by the Human/Mouse/Rat Phospho-JNK2 (T183/Y185) DuoSet IC ELISA, are consistent with the amounts of phosphorylated JNK1, JNK2, and JNK3 determined by qualitative Western blot analysis.

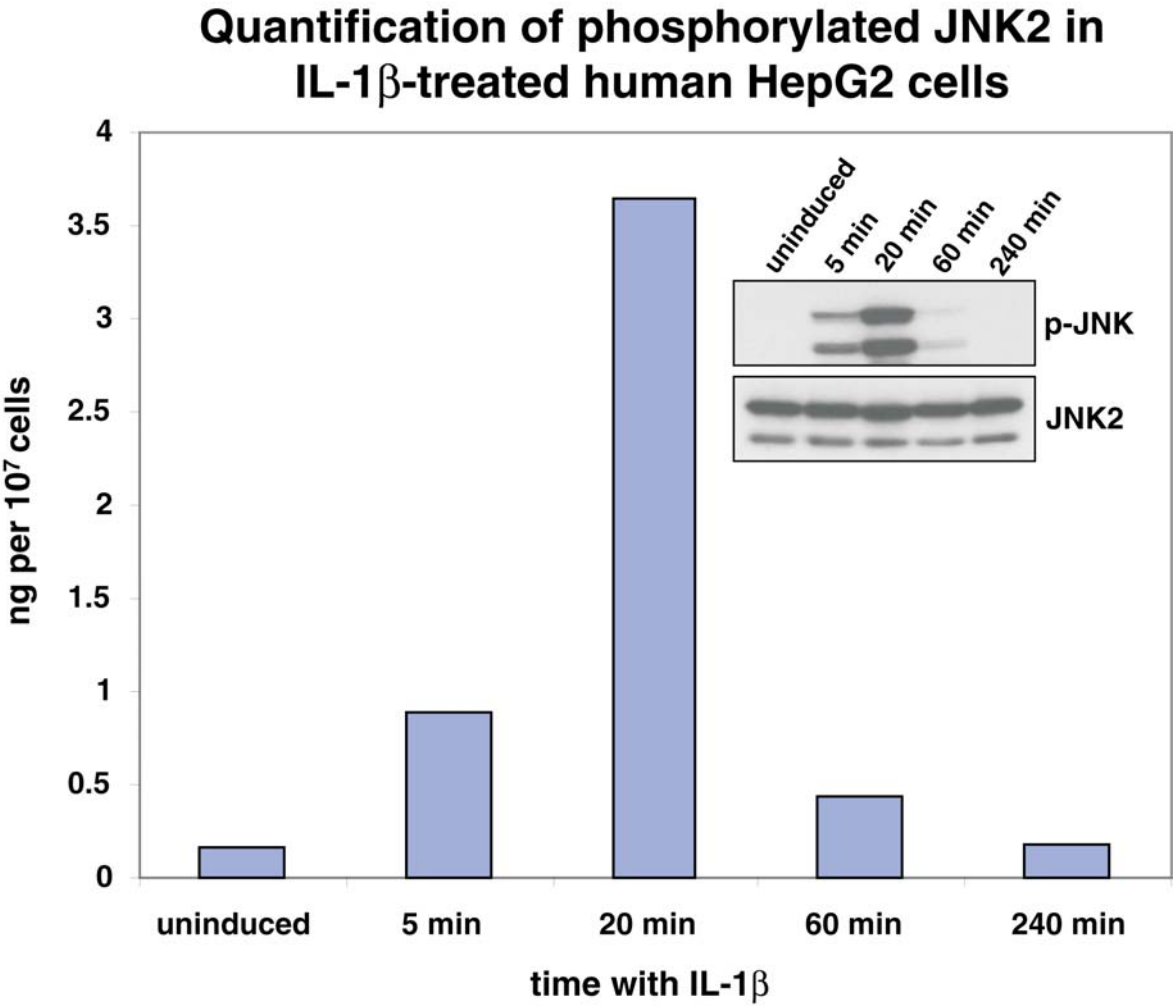


Figure 2: Lysates prepared from HepG2 human hepatocellular carcinoma cells induced with IL-1β for the indicated times were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-pan JNK (p-JNK) (R&D Systems, Catalog # AF1205) or anti-JNK2 (R&D Systems, Catalog # MAB1846) antibodies. The DuoSet IC ELISA results are consistent with the overall phosphorylation of JNK1, JNK2, and JNK3 detected by Western blot. The immunoblot with anti-JNK2 antibody indicates that total levels of JNK2 remained constant during the induction with IL-1β.

The quantification of phosphorylated JNK2 with this DuoSet IC ELISA was also determined using cells pretreated with a selective pan JNK inhibitor SP600125 (Tocris, Catalog # 1496), which directly inhibits the activity of JNK1, JNK2, and JNK3 and also diminishes their phosphorylation.

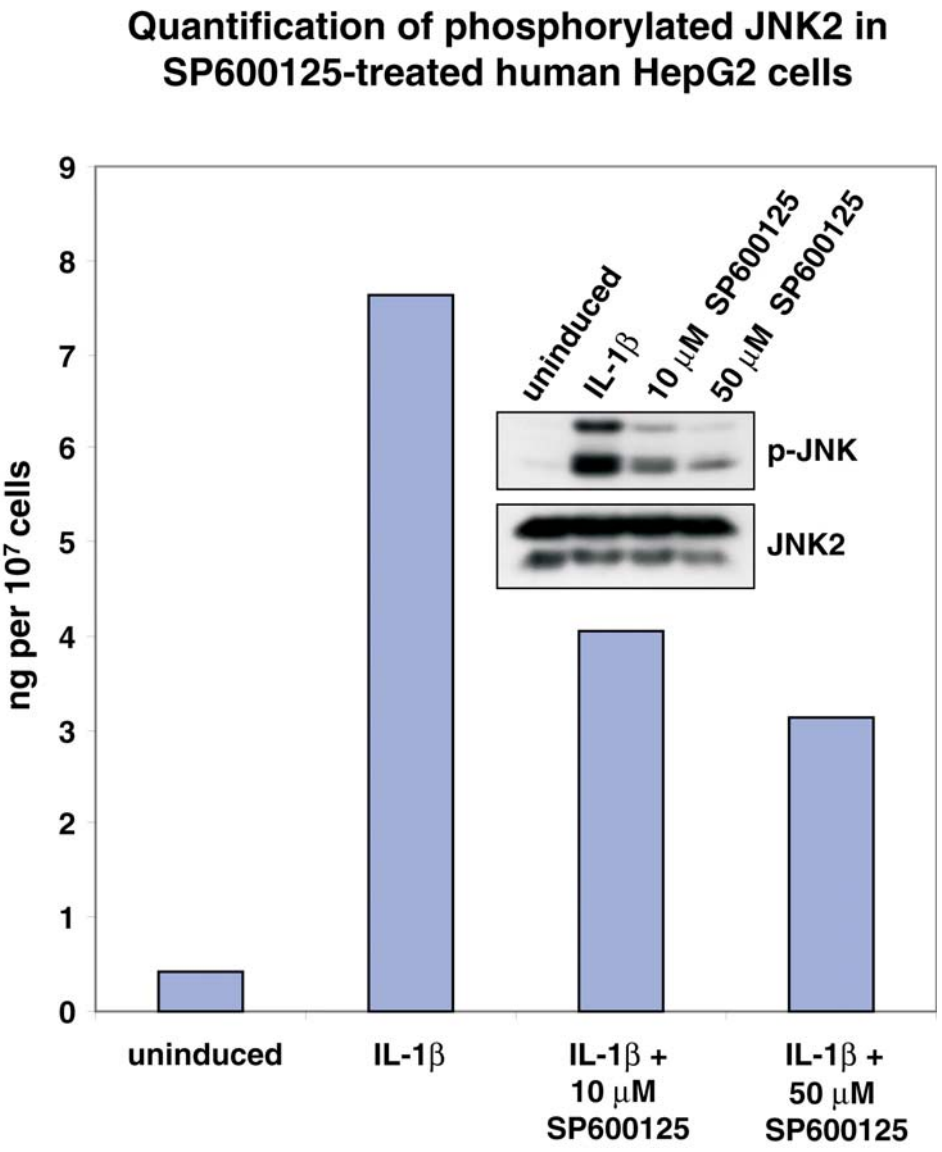


Figure 3: HepG2 human hepatocellular carcinoma cells were incubated with no additions or with IL-1β for 20 minutes, either with or without SP600125 at the indicated concentrations. Cells were lysed and phosphorylated JNK2 was quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-pan JNK (p-JNK) or anti-JNK2 antibodies. The DuoSet IC ELISA results are consistent with the overall phosphorylation of JNK1, JNK2, and JNK3 detected by Western blot. The immunoblot with anti-JNK2 antibody indicates that total levels of JNK2 remained constant during the various treatments.

The Human/Mouse/Rat Phospho-JNK2 (T183/Y185) DuoSet IC ELISA also quantifies phosphorylated JNK2 levels in mouse and rat cell lysates.

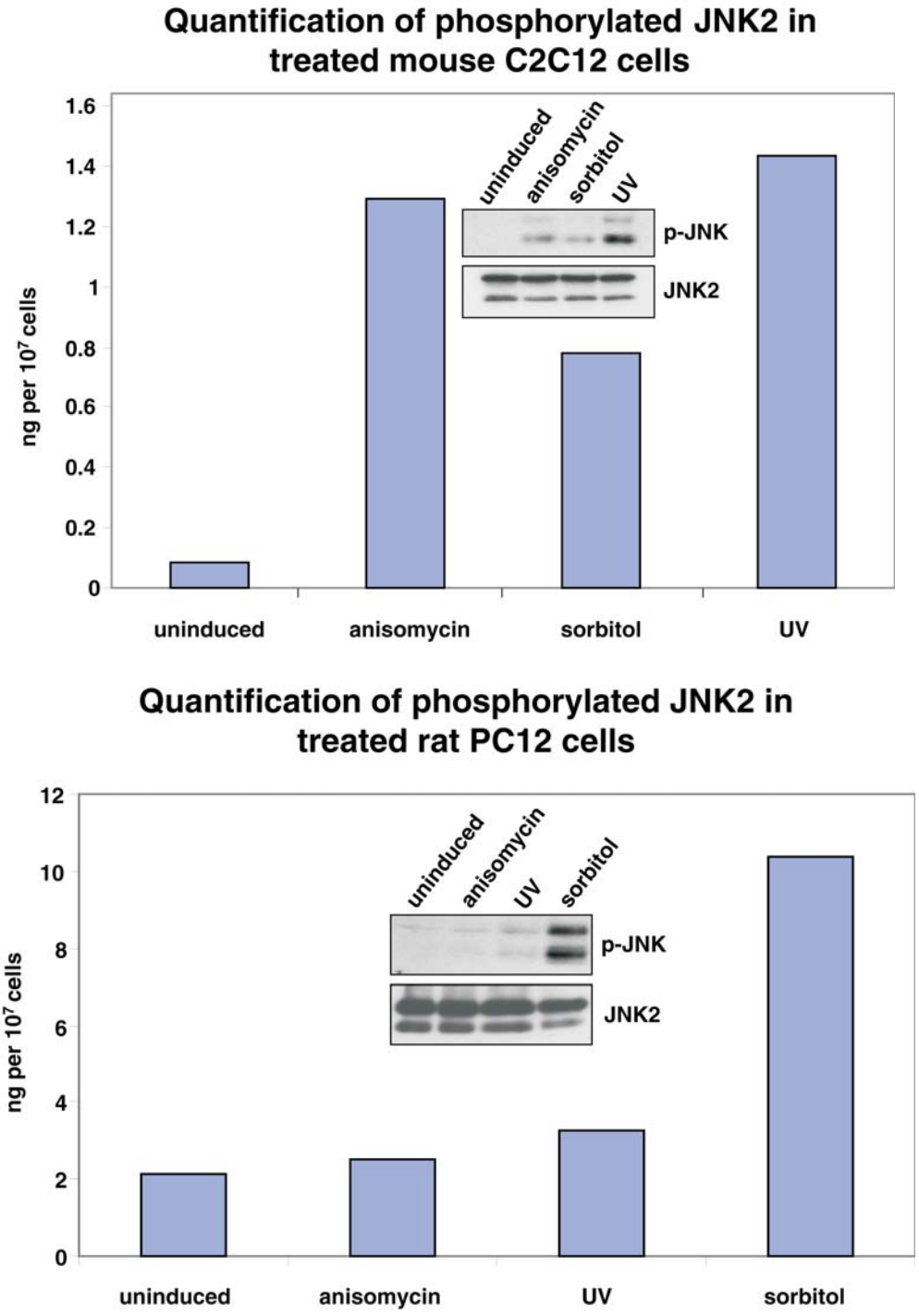


Figure 4: Lysates prepared from C2C12 mouse myoblast cells (top panel) and PC-12 rat adrenal pheochromocytoma cells (bottom panel), either uninduced or induced with 25 μg/mL anisomycin, 300 mM sorbitol, or 200 J/m^2 UV, were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-pan JNK (p-JNK) or anti-JNK2 antibodies. The DuoSet IC ELISA results are consistent with the overall phosphorylation of JNK1, JNK2, and JNK3 detected by Western blot. The immunoblot with anti-JNK2 antibody indicates that total levels of JNK2 remained constant during the various inductions.