DuoSet® IC

Human/Mouse/Rat Phospho-JNK Pan Specific

Catalog Number DYC1387-2
DYC1387-5
DYC1387E

For the development of sandwich ELISAs to measure phosphorylated JNK 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 phospho-JNK in cell lysates. An immobilized capture antibody specific for JNK1, JNK2, and JNK3 (also known as SAPK1γ, SAPK1α, and SAPK1β; and MAPK8, MAPK9, and MAPK10) binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody specific for JNK1 and JNK2 dually phosphorylated at T183/Y185, and JNK3 dually phosphorylated at T221/Y223, is used to detect only phosphorylated protein, utilizing a standard Streptavidin-HRP format.

MATERIALS PROVIDED

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

			Vials Provided	
Description	**Part #**	**Storage Conditions**	**Cat. # DYC1387-2**	**Cat. # DYC1387-5**
Phospho-JNK Pan Specific Capture Antibody	841461	2-8° C	1	2
Phospho-JNK Pan Specific Detection Antibody	841462	2-8° C	1	2
Phospho-JNK Pan Specific Standard	841463	2-8° C	3	5
Streptavidin-HRP	890803	2-8° C	1	1

DYC1387-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC1387-5 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 # DYC1387E). 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 # P6508), 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,* 0.05% NaN_3, in PBS, pH 7.2-7.4.

IC Diluent #1 - 1% BSA* in PBS, pH 7.2-7.4, 0.2 μm filtered (R&D Systems, Catalog # DY995).

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 buffer 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, 10 μg/mL Leupeptin, 10 μg/mL Pepstatin, 100 μM PMSF, 3 μg/mL Aprotinin, 2.5 mM sodium pyrophosphate, 1 mM activated sodium orthovanadate 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 package insert.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Phospho-JNK Pan Specific Capture Antibody (Part 841461) - Each vial contains 720 µg/mL of goat anti-human JNK Pan Specific 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-JNK Pan Specific Detection Antibody (Part 841462) - Each vial contains 18 µg/mL of biotinylated rabbit anti-human phospho-JNK Pan Specific 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-JNK Pan Specific Standard (Part 841463) - Each vial contains 210 ng/mL of recombinant human phospho-JNK1 when reconstituted with 500 µL of IC Diluent #7. **Use within one hour of reconstitution. A fresh standard should be used for each assay.** An initial 6-fold dilution should be made in IC Diluent #8. Further dilutions should be made in IC Diluent #3 immediately before use. A seven point curve using 2-fold serial dilutions and a high standard of 20 ng/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. Vortex lysates briefly and allow to sit on ice for 15 minutes before use or store at ≤ -20° C in a manual defrost freezer. Sample protein concentration may be quantified using a total protein assay. Before use, centrifuge at 2000 x g for 5 minutes and transfer the supernate into a clean test tube. 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 and standards should be 1 M prior to addition 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

A plate layout is provided to record standards and samples assayed.

Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 4.0 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 20 ng/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 0.5 μg/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.

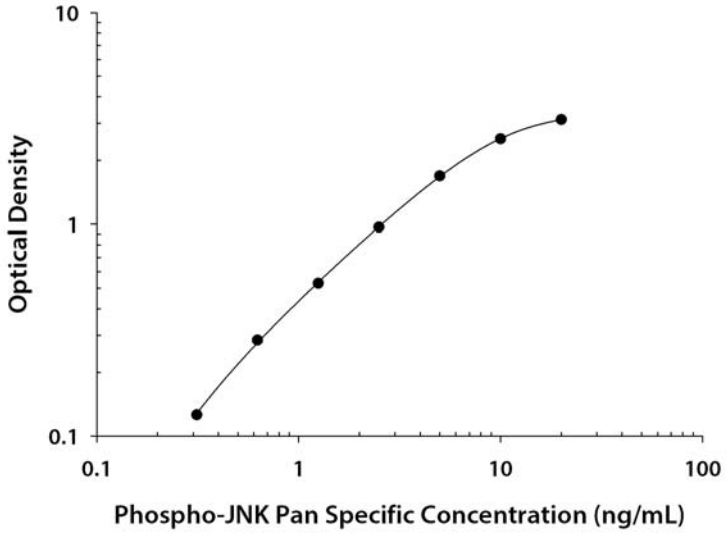
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and 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-JNK Pan Specific 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-JNK Pan Specific 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-JNK Pan Specific DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human Phospho-JNK1 produced at R&D Systems. Samples containing natural phospho-JNK showed linear dilution parallel to the standard curve obtained using the Phospho-JNK Pan Specific Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the relative concentration of phospho-JNK in natural samples.

SPECIFICITY

The Human/Mouse/Rat Phospho-JNK Pan Specific DuoSet IC ELISA specifically recognizes JNK1 and JNK2 dually phosphorylated at T183/Y185, and JNK3 dually phosphorylated at T221/Y223. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody supplied in the kit.

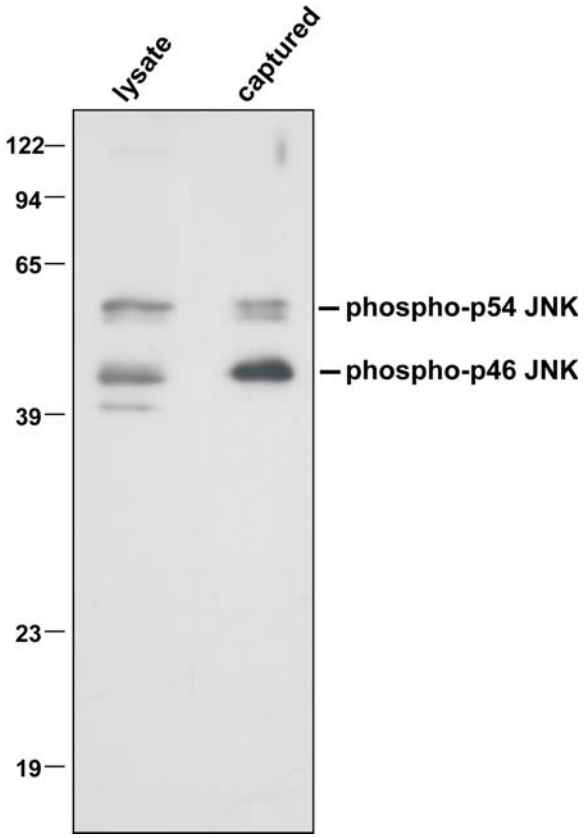


Figure 1: Lysates prepared from HepG2 human hepatocellular carcinoma cells treated with 10 ng/mL of recombinant human IL-1β (R&D Systems, Catalog # 201-LB) were incubated in wells coated with the Phospho-JNK Pan Specific Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to a PVDF membrane and immunoblotted with the Phospho-JNK Pan Specific Detection Antibody. Two bands corresponding to phosphorylated p46 and p54 JNK, the predominant isoforms of JNK detected by Western blot, were found in captured material and lysates.

To further determine specificity, unphosphorylated recombinant human JNK1 was assayed at 100 ng/mL and read 163 pg/mL (0.2% cross-reactivity). Unphosphorylated recombinant human JNK2 and unphosphorylated recombinant human JNK3 were assayed at 100 ng/mL and did not cross-react. Other MAP Kinases, unphosphorylated recombinant human p38α and unphosphorylated recombinant human ERK2, were assayed at 100 ng/mL and did not cross-react in the assay.

QUANTIFICATION

Amounts of human phosphorylated JNK, as quantified by the Phospho-JNK Pan Specific DuoSet IC ELISA, are consistent with the relative amounts of phosphorylated JNK determined by qualitative Western blot analysis.

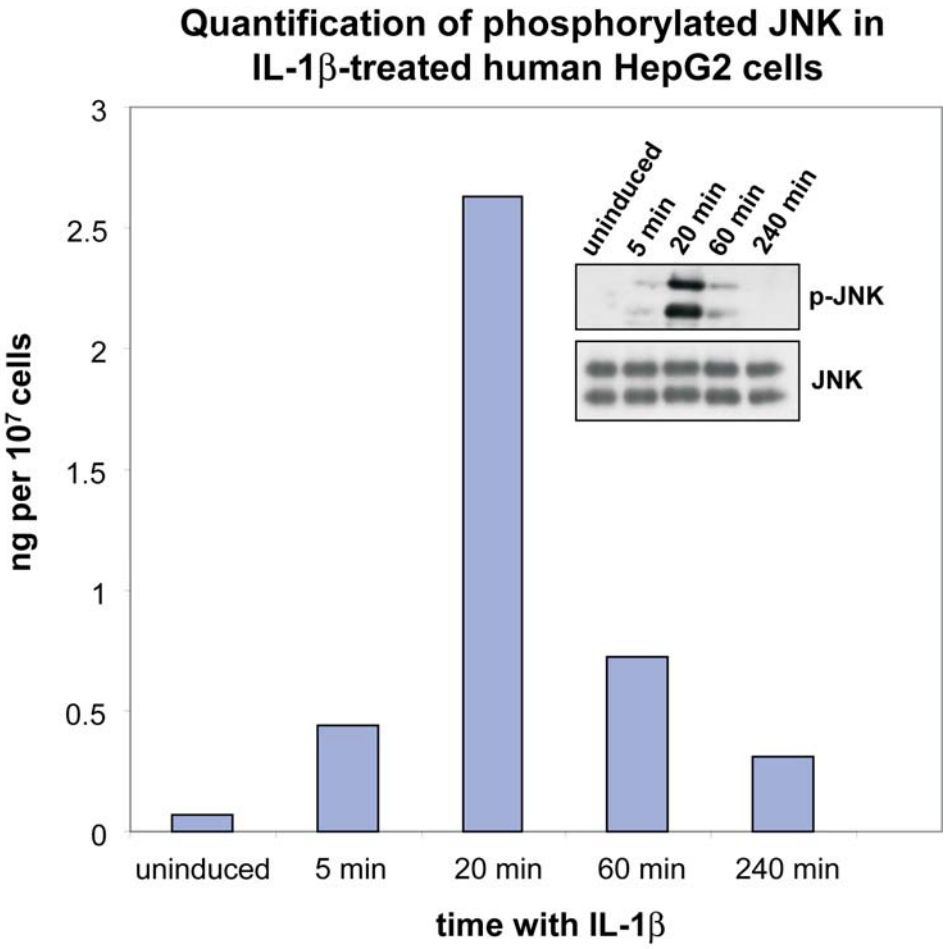


Figure 2: HepG2 human hepatocellular carcinoma cells were treated with 10 ng/mL of IL-1β for the indicated times. Following cell lysis, phosphorylated JNK1, JNK2, and JNK3 were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-JNK (p-JNK) (R&D Systems, Catalog # AF1205) or anti-JNK Pan Specific (R&D Systems, Catalog # AF1387) polyclonal antibodies. The DuoSet IC ELISA results correlate well with the relative amounts of phosphorylated p46 and p54 JNK detected by Western blot. The blot with anti-JNK Pan Specific antibody indicates that total levels of JNK remained constant during incubations with IL-1β.

The Phospho-JNK Pan Specific DuoSet IC ELISA also quantifies phosphorylated JNK levels in mouse and rat cell lysates.

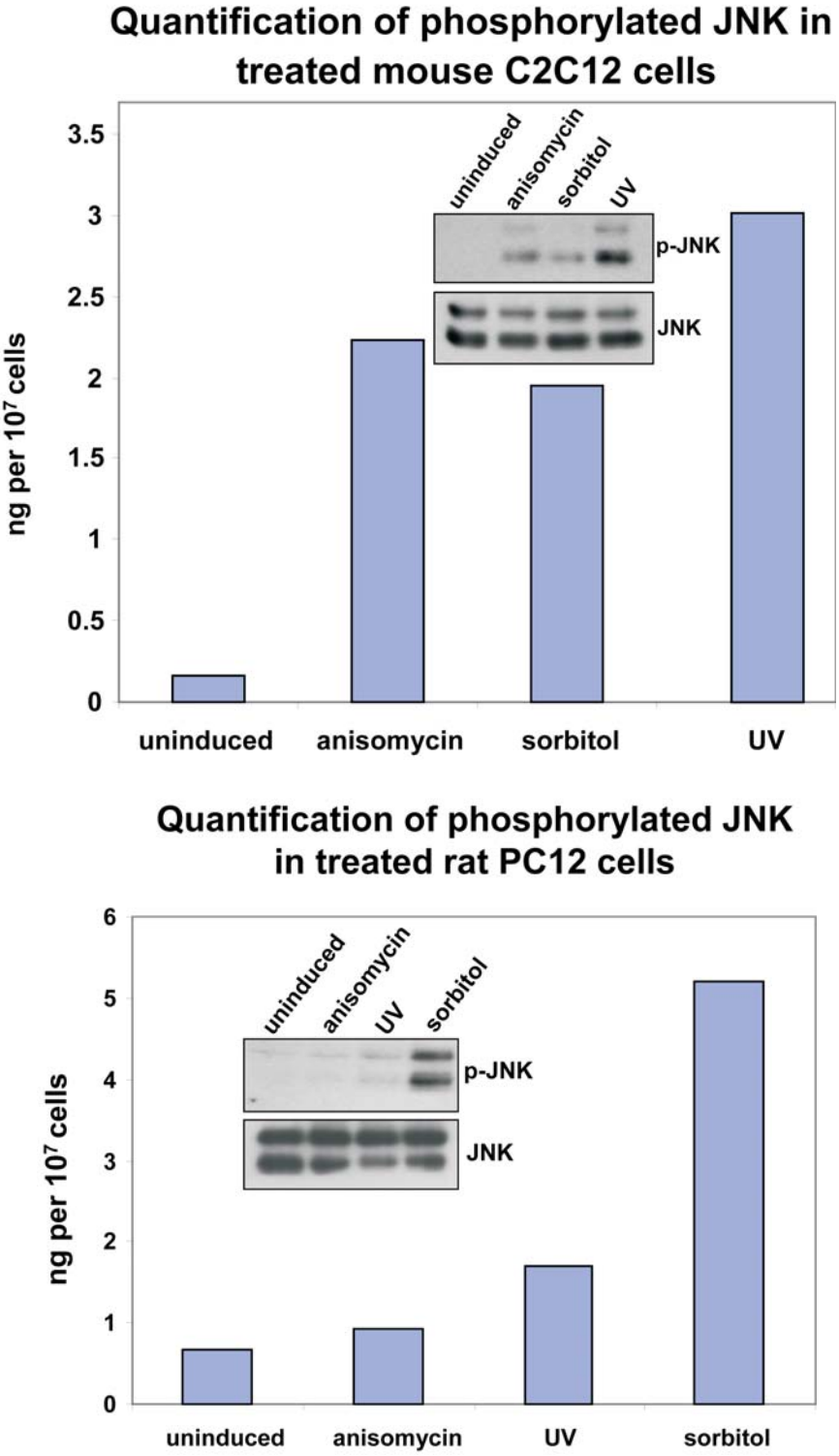


Figure 3: Lysates prepared from C2C12 mouse myoblast cells (top panel) and PC-12 rat adrenal pheochromocytoma cells (bottom panel), either uninduced or induced with 25 μg/mL anisomycin, 300 mM sorbitol, or 200 J/m^2 UV, were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-JNK (p-JNK) or anti-JNK polyclonal antibodies. The DuoSet IC ELISA results correlate well with the relative amounts of phosphorylated p46 and p54 JNK detected by Western blot. The blots with anti-JNK antibody indicates that total levels of JNK remained constant during the various inductions.

PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.