Surveyor™ IC

Human/Mouse/Rat Phospho-p38α (T180/Y182) Immunoassay

Catalog Number SUV869B

For the quantitative determination of p38α phosphorylated at T180/Y182 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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INTRODUCTION

The p38 mitogen-activated protein kinases (p38 MAP kinases) are a family of four related Ser/Thr kinases responsive to pro-inflammatory cytokines and environmental stresses, including ionizing radiation, oxidative stress, and osmotic shock (1). Each family member, p38α, p38β, p38δ, and p38γ, is activated by dual Thr and Tyr phosphorylation within a Thr-Gly-Tyr motif residing in the kinase activation loop. For p38α, also known as stress-activated protein kinase 2A (SAPK2A), CSAID-binding protein 1 (CSBP1), and MAPK14, this dual phosphorylation occurs at Thr180/Tyr182 (2). The MAP kinase kinases MKK3 and MKK6 phosphorylate the four p38 isoforms with varying selectivity, helping to confer different functions among family members (3). Functional differences are also contributed by tissue-specific p38 expression. Whereas p38α is ubiquitously expressed, p38β is expressed at highest levels in the brain and heart, p38δ is enriched in endocrine organs, and p38γ is expressed primarily in skeletal muscle (4).

Active p38 phosphorylates cytokine- and stress-responsive proteins in both the cytosol and nucleus. Cytoplasmic targets include the kinases PRAK (5) and MAPKAPK-2, which both phosphorylate the thermotolerance chaperone HSP27 (6). Nuclear targets include the transcription factors ATF2 (7), Max (8), and STAT1 (9). The p38 MAP kinases also regulate transcription indirectly by phosphorylating kinases from the MSK family, which activate the transcription factor CREB (10). Several promising compounds that inhibit p38 activity have entered clinical trials as potential therapies for arthritis, asthma, and other inflammatory diseases (11).

PRINCIPLE OF THE ASSAY

This Surveyor IC Immunoassay employs a two-site sandwich ELISA to quantitate p38α phosphorylated at T180/Y182 in cell lysates. An antibody specific for p38α, binding both phosphorylated and unphosphorylated protein, has been pre-coated onto a microplate. Standards and samples are added and p38α present is bound by the immobilized antibody. After washing away unbound material, a biotinylated detection antibody recognizing p38α dually phosphorylated at T180 and Y182 is used to detect only phosphorylated protein, utilizing a standard streptavidin-HRP format. Substrate Solution is added to the wells and color develops in proportion to the amount of phosphorylated p38α bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Surveyor IC Immunoassay should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Assay Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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 remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Phospho-p38α (T180/Y182) Microplate	843886	One 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against p38α.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Phospho-p38α (T180/Y182) Standard	840772	2 vials (110 ng/vial) of recombinant human phospho-p38α (T180/Y182) in a buffered protein base with preservatives; lyophilized.	Use within 1 hour of reconstitution. Use a fresh standard for each assay.
Phospho-p38α (T180/Y182) Detection Antibody	841829	6.0 µg of a biotinylated rabbit anti-phospho-p38α (T180/Y182) antibody; lyophilized.	May be stored for up to 1 month at 2-8 °C.*
Lysis Buffer 6	895561	21 mL of a cell lysing buffer with phosphatase inhibitors and preservatives.	
Sample Diluent Concentrate 1 (5X)	895562	21 mL of a 5-fold concentrated buffer with preservatives.	
Reagent Diluent Concentrate 2 (10X)	841380	21 mL of a 10-fold concentrated solution of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Streptavidin-HRP	890803	1.0 mL of streptavidin conjugated to horseradish-peroxidase.	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- 100 mL and 500 mL graduated cylinders.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Phosphate-buffered saline (PBS).
- Phenylmethylsulfonylfluoride (PMSF) (optional; Sigma, Catalog # P7626).
- Protease Inhibitor Cocktail (optional; Sigma, Catalog # P2714).
- **Polypropylene** test tubes for diution of standards and samples.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Sample Diluent 1 - Add 20 mL of Sample Diluent Concentrate 1 (5X) to deionized or distilled water to prepare 100 mL of Sample Diluent 1.

Assay Diluent - Add 8 mL of Lysis Buffer 6 to Sample Diluent 1 to prepare 48 mL of Assay Diluent. Prepare only enough diluent to run the assay.

Reagent Diluent 2 - Add 5 mL of Reagent Diluent Concentrate 2 (10X) to deionized or distilled water to prepare 50 mL of Reagent Diluent 2.

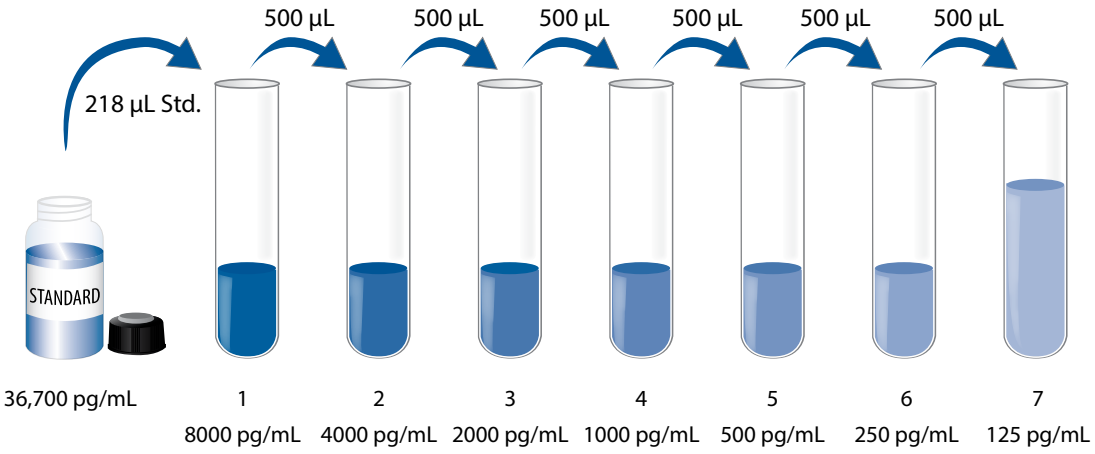
Phospho-p38α (T180/Y182) Detection Antibody - Reconstitute the Phospho-p38α (T180/Y182) Detection Antibody with 1.0 mL Reagent Diluent 2. This reconstitution produces a stock solution of 6.0 µg/mL. Immediately before use, dilute the Detection Antibody to a working concentration of 400 ng/mL in Reagent Diluent 2.

Streptavidin-HRP - Immediately before use, dilute Streptavidin-HRP to the working concentration specified on the vial label using Reagent Diluent 2.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Prepare only enough Substrate Solution as needed to run the assay. Protect from light. 100 µL of the resultant mixture is required per well.

Phospho-p38α (T180/Y182) Standard - Reconstitute the Phospho-p38α (T180/Y182) Standard with 0.5 mL of Lysis Buffer 6. This reconstitution produces a stock solution of 220 ng/mL. Mix the standard to ensure complete reconstitution. **Allow the standard to sit for a minimum of 15 minutes.** Perform a 6-fold dilution of the Standard by adding 2.5 mL of Sample Diluent 1 to the vial. This dilution produces a stock solution of 36,700 pg/mL.

Label seven **polypropylene** tubes 1 through 7. Add 782 µL of Assay Diluent into tube 1 and 500 µL of Assay Diluent into tubes 2 through 7. Add 218 µL of the 36,700 pg/mL stock Standard to tube 1. Mix thoroughly and continue to prepare a seven point standard curve using 2-fold serial dilutions by transferring 500 µL from tube 1 into tube 2 and subsequent 500 µL transfers as shown below. Use Assay Diluent as the zero standard. **Use a fresh standard for each assay. Use within 1 hour of preparation.**



CELL LYSIS PROCEDURE

Note: *It is recommended to supplement Lysis Buffer 6 with PMSF and the Protease Inhibitor Cocktail prior to use. Supplements should be used according to the manufacturer's instructions.*

1. Using PBS, collect non-adherent cells by centrifugation and adherent cells by scraping the culture flask.
2. Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse.
3. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer 6.
4. Vortex lysates briefly and allow to sit on ice for 15 minutes or store at ≤ -20 °C in a manual defrost freezer. Sample protein concentration may be quantified using a total protein assay.
5. Before use, centrifuge at 2000 x g for 5 minutes and transfer the supernates into a clean test tube.
6. For assaying, dilute lysates 6-fold with Sample Diluent 1 and make further serial dilutions in Assay Diluent.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Standard or sample* per well. Use Assay Diluent as the zero standard. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
4. Aspirate each well and wash, repeating the process two times for a total of three 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 to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Immediately before use, dilute the Detection Antibody. Add 100 µL of diluted Phospho-p38α (T180/Y182) Detection Antibody to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
6. Repeat the aspiration/wash as in step 4.
7. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using Reagent Diluent 2. Add 100 µL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature.
8. Repeat the aspiration/wash as in step 4.
9. Immediately before use, prepare the Substrate Solution. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Avoid placing the plate in direct light.**
10. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, 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.

*Lysates require dilution. Refer to the Cell Lysis Procedure.

CALCULATION OF RESULTS

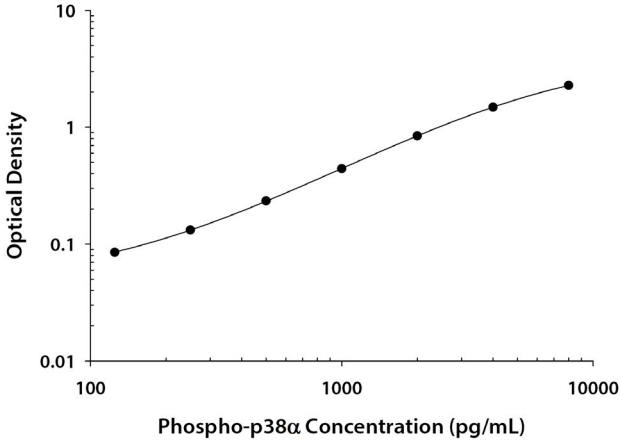
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

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-p38α (T180/Y182) 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.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

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-p38α (T180/Y182) Surveyor IC Immunoassay. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is provided for demonstration purposes only.



(pg/mL)	O.D.	Corrected	Average
0	0.096	—	—
	0.096		
125	0.171	0.075	0.085
	0.190	0.094	
250	0.216	0.120	0.131
	0.238	0.142	
500	0.324	0.228	0.234
	0.335	0.239	
1000	0.512	0.416	0.441
	0.562	0.466	
2000	0.870	0.774	0.844
	1.008	0.912	
4000	1.557	1.461	1.484
	1.603	1.507	
8000	2.321	2.225	2.275
	2.421	2.325	

CALIBRATION

The Human/Mouse/Rat Phospho-p38α (T180/Y182) Surveyor IC Immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-p38α (T180/Y182) produced at R&D Systems. Samples containing natural phospho-p38α (T180/Y182) showed linear dilution parallel to the standard curve obtained using the Phospho-p38α (T180/Y182) Standard. These results indicate that O.D. values from this Surveyor IC Immunoassay can be used to determine the relative concentration of phospho-p38α (T180/Y182) in natural samples.

SPECIFICITY

The Human/Mouse/Rat Phospho-p38α (T180/Y182) Surveyor IC Immunoassay specifically recognizes p38α phosphorylated at T180/Y182. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody coated on the plate and cross-reactivity analysis.

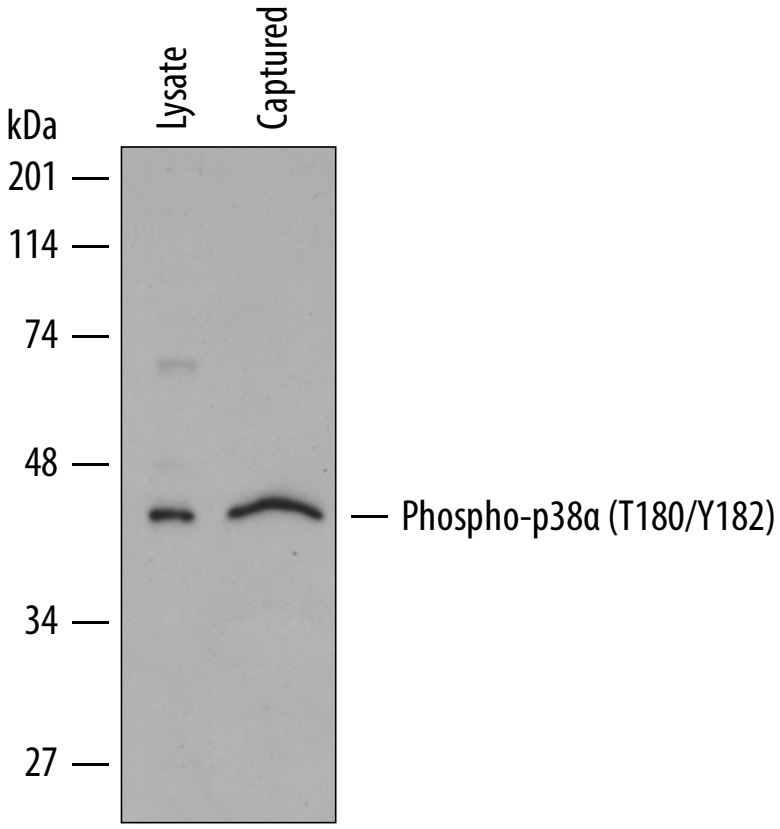


Figure 1: Lysates prepared from HepG2 human hepatocellular carcinoma cells treated with 10 ng/mL of recombinant human (rh) IL-1β (R&D Systems, Catalog # 201-LB) for 20 minutes were incubated in wells coated with Phospho-p38α (T180/Y182) Capture Antibody. Unbound material was removed by washing and bound 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-p38α (T180/Y182) Detection Antibody. Only a single band corresponding to phosphorylated p38α was detected in captured material.

Cross-reactivity experiments were performed with this Surveyor IC Immunoassay to further determine specificity. Unphosphorylated rhp38α, rhp38β, rhp38δ and rhp38γ, and rhp38δ phosphorylated at T180/Y182 and rhp38γ phosphorylated at T183/Y185, were assayed at 80 ng/mL and did not cross-react or interfere in the assay.

QUANTIFICATION

Amounts of phosphorylated p38α, as quantified by the Human/Mouse/Rat Phospho-p38α (T180/Y182) Surveyor IC Immunoassay, are consistent with the relative amounts of phosphorylated p38α determined by qualitative Western blot analysis.

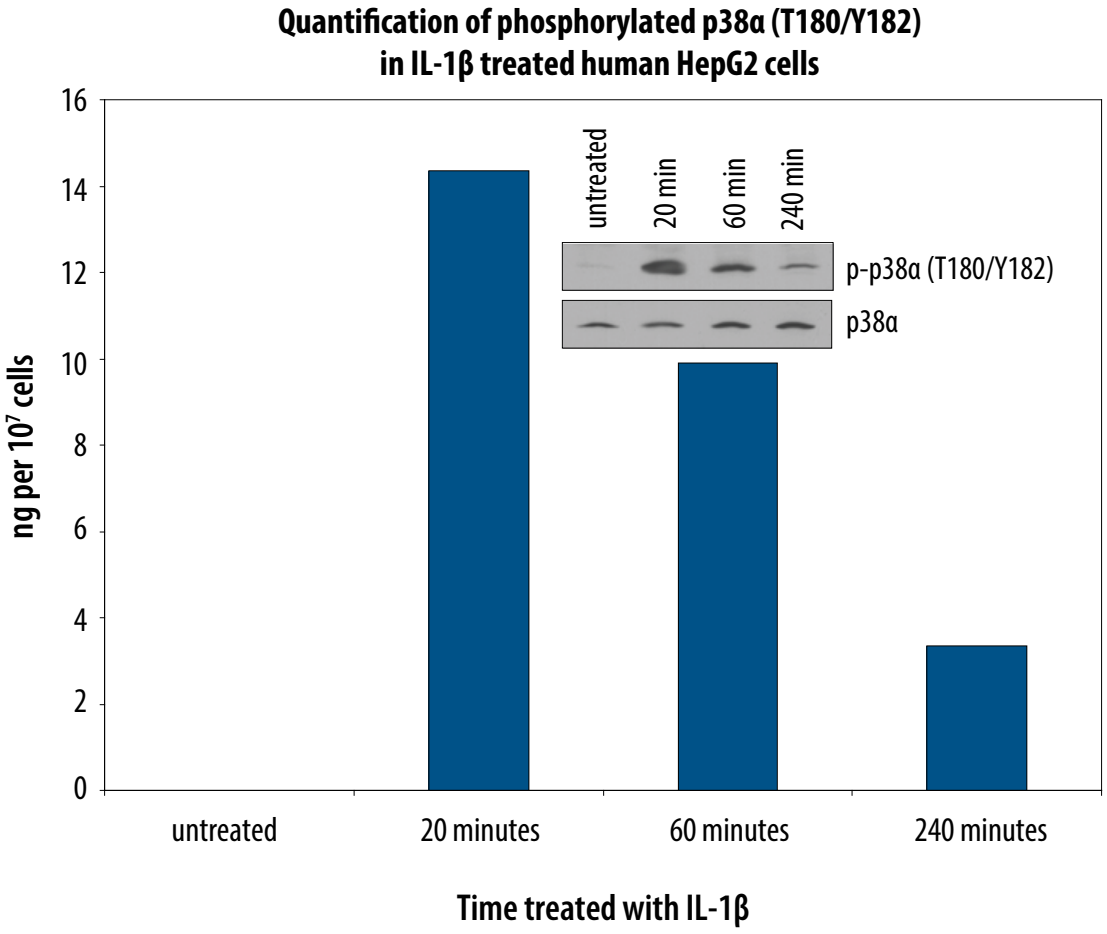


Figure 2: Lysates prepared from HepG2 human hepatocellular carcinoma cells treated with 10 ng/mL rhIL-1β for the indicated times were quantified with this Surveyor IC Immunoassay. The same lysates were also immunoblotted (inset) with either anti-phospho-p38 MAP Kinase (T180/Y182) (R&D Systems, Catalog # AF869) or anti-p38α (R&D Systems, Catalog # MAB869) antibodies. The Surveyor IC Immunoassay results correlate well with the relative amounts of phosphorylated p38α detected by Western blot. The immunoblot with anti-p38α antibody indicates that total levels of p38α remained constant during the induction.

QUANTIFICATION *CONTINUED*

The Human/Mouse/Rat Phospho-p38α (T180/Y182) Surveyor IC Immunoassay also quantifies phosphorylated p38α levels in mouse and rat cell lysates.

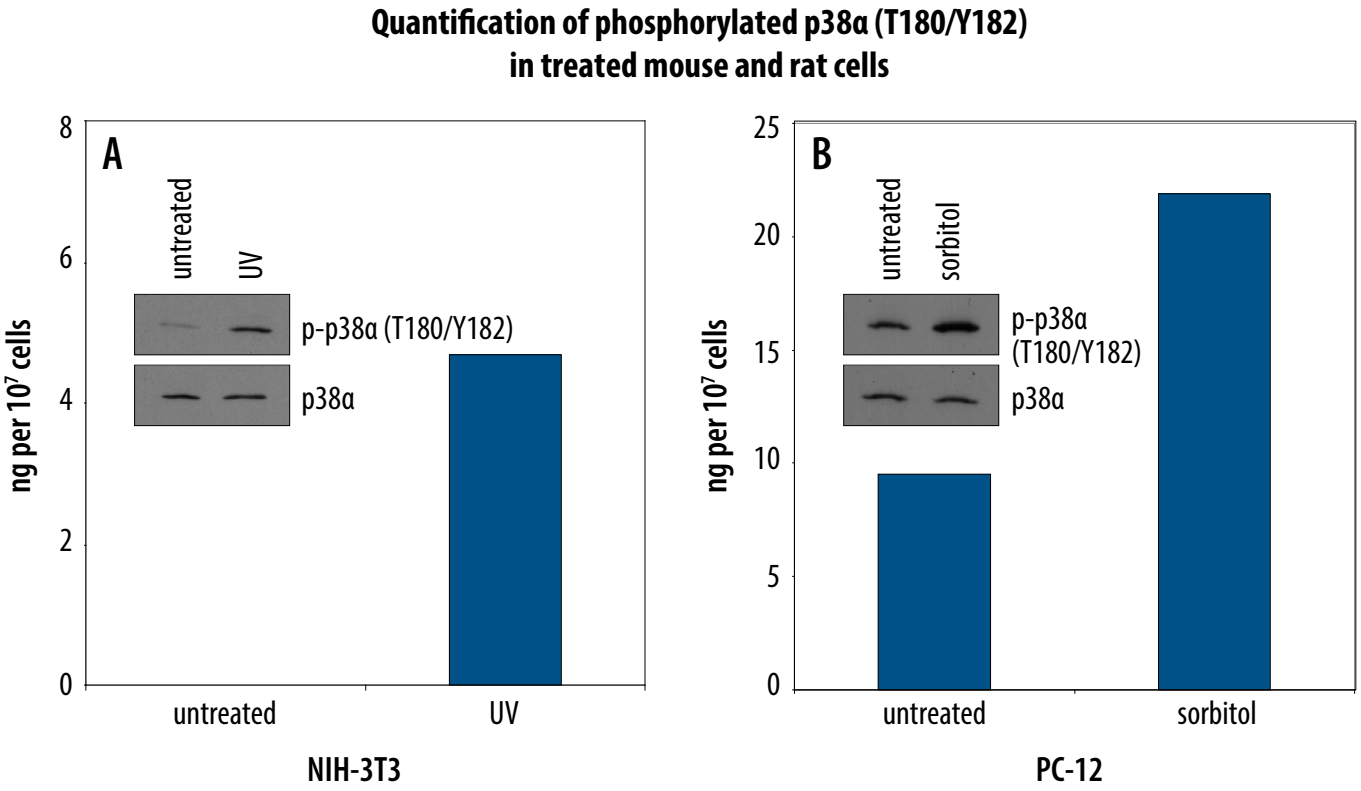


Figure 3: Lysates prepared from **(A)** NIH-3T3 mouse embryonic fibroblast cells either untreated or treated with 20 mJ/cm^2 UV, and **(B)** PC-12 rat adrenal pheochromocytoma cells either untreated or treated with 300 mM sorbitol for 30 minutes were quantified with this Surveyor IC Immunoassay. The same lysates were also immunoblotted (inset) with either anti-phospho-p38 MAP Kinase (T180/Y182) or anti-p38α antibodies. The Surveyor IC Immunoassay results correlate well with the relative amounts of phosphorylated p38α detected by Western blot. The immunoblots with anti-p38α antibody indicate that total levels of p38α remained constant during the induction.

REFERENCES

1. Kyriakis, J.M. and J. Avruch (2001) Physiol. Rev. **81**:807.
2. Raingeaud, J. *et al.* (1995) J. Biol. Chem. **270**:7420.
3. Enslen, H. *et al.* (1998) J. Biol. Chem. **273**:1741.
4. Wang, X.S. *et al.* (1997) J. Biol. Chem. **272**:23668.
5. New, L. *et al.* (1998) EMBO J. **17**:3372.
6. Freshney, N.W. *et al.* (1994) Cell **78**:1039.
7. Cuenda, A. *et al.* (1997) EMBO J. **16**:295.
8. Zervos, A.S. *et al.* (1995) Proc. Natl. Acad. Sci. USA **92**:10531.
9. Kovarik, P. *et al.* (1999) Proc. Natl. Acad. Sci. USA **96**:13956.
10. Arthur, J.S.C. *et al.* (2004) J. Neurosci. **24**:4324.
11. Kumar, S. *et al.* (2003) Nat. Rev. Drug Discov. **2**:717.