Surveyor™ IC

Human/Mouse/Rat Phospho-HSP27 (S78/S82) Immunoassay

Catalog Number SUV2314

For the quantitative determination of Heat Shock Protein 27 (HSP27) phosphorylated at S78/S82 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.

TABLE OF CONTENTS

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Heat Shock Proteins (HSPs) are molecular chaperones that facilitate the proper folding of various polypeptides, regulate protein degradation, and assist with protein translocation to different cellular compartments (1). HSP27, also known as HSP25, HSPB1, and SRP27, is a member of the small heat shock protein (sHSP) family. Induced by stress conditions, HSP27 not only functions as a modulator of cellular thermotolerance (2) and chemoresistance (3) but acts as an inhibitor of actin polymerization (4), a stabilizer of RNA (5), and a regulator of apoptosis (6).

HSP27 expression is regulated both transcriptionally and post-translationally. Transcriptional induction occurs upon the binding of pre-existing heat shock transcription factors (HSFs) to conserved heat shock elements (7), while phosphorylation of human HSP27 at S15, S78, and S82 follows the stimulation of the p38 MAP kinase cascade and is catalyzed by MAPKAP kinase 2 (8). Cellular stresses induce HSP27 phosphorylation within minutes, in addition to initiating an increase in HSP27 mRNA and protein levels detectable after several hours (9). Mutant unphosphorylatable HSP27 is much less effective than wild-type HSP27 in protecting cells from heat shock or oxidative stress, indicating the importance of HSP27 phosphorylation (10). The serine/threonine kinases MAPKAP kinase 5/PRAK (11) and cGMP-dependent protein kinase (12) have also been implicated in the phosphorylation of HSP27.

PRINCIPLE OF THE ASSAY

This Surveyor IC Immunoassay employs a two-site sandwich ELISA to quantitate HSP27 phosphorylated at S78/S82 in cell lysates. An antibody specific for HSP27, binding both phosphorylated and unphosphorylated protein, has been pre-coated onto a microplate. Standards and samples are added and any HSP27 present is bound by the immobilized antibody. After washing away unbound material, a biotinylated detection antibody recognizing human HSP27 dually phosphorylated at S78 and S82, and mouse/rat HSP27 singly phosphorylated at S86 (where N82 replaces the residue analogous to S78 of the human sequence) 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 HSP27 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 Sample Diluent 1 (diluted 1:5) 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-HSP27 (S78/S82) Microplate	842718	96 well polystyrene microplate (12 strips of 8 wells) coated with a goat polyclonal antibody against HSP27.	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-HSP27 (S78/S82) Standard	841759	2 vials (90 ng/vial) of recombinant human phospho-HSP27 (S78/S82) in a buffered protein base with preservatives; lyophilized.	Use within 1 hour of reconstitution. Use a fresh standard for each assay.
Phospho-HSP27 (S78/S82) Detection Antibody	842719	7.5 µg of a biotinylated rabbit anti-phospho-HSP27 (S78/S82) polyclonal antibody; lyophilized.	May be stored for up to 1 month at 2-8 °C.*
Sample Diluent Concentrate 1 (5X)	895562	2 vials (21 mL/vial) of a concentrated buffer with preservatives. *Used diluted 1:5 in this assay.*	
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 (diluted 1:5) - Add 20 mL of Sample Diluent Concentrate 1 (5X) to deionized or distilled water to prepare 100 mL of Sample Diluent 1 (diluted 1:5). 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.

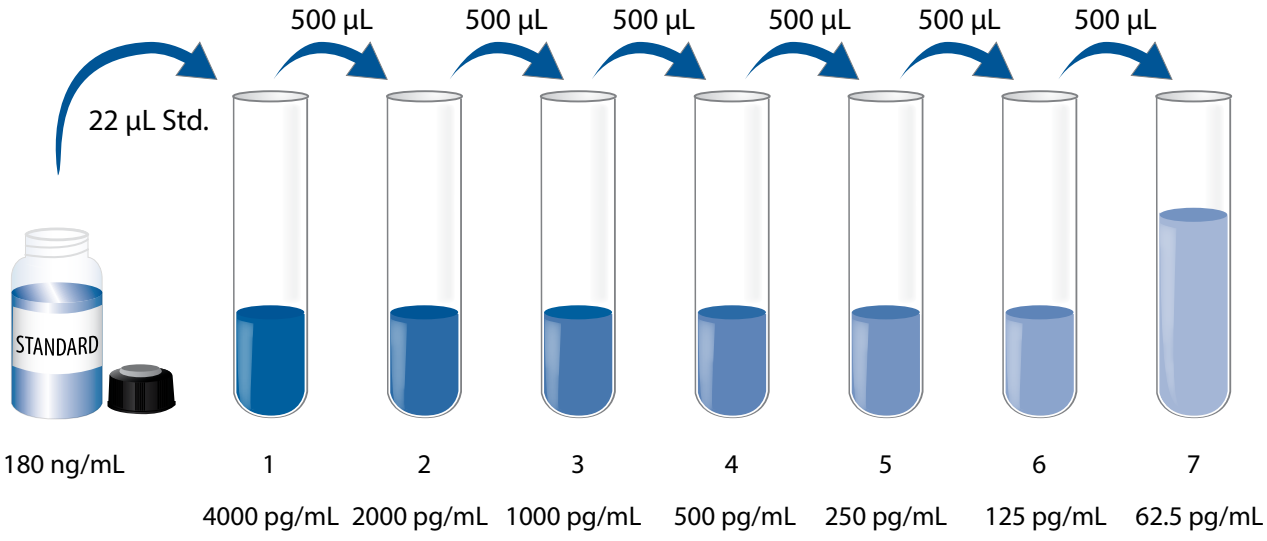
Phospho-HSP27 (S78/S82) Detection Antibody - Reconstitute the Phospho-HSP27 (S78/S82) Detection Antibody with 1.0 mL Reagent Diluent 2. This reconstitution produces a stock solution of 7.5 µg/mL. Immediately before use, dilute the Detection Antibody to a working concentration of 0.5 µg/mL in Reagent 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-HSP27 (S78/S82) Standard - Reconstitute the Phospho-HSP27 (S78/S82) Standard with 0.5 mL of Assay Diluent. This reconstitution produces a stock solution of 180 ng/mL. Mix the standard to ensure complete reconstitution. **Allow the standard to sit for a minimum of 15 minutes.**

Label seven **polypropylene tubes** 1 through 7. Add 978.8 µL of Sample Diluent 1 (diluted 1:5) into tube 1 and 500 µL of Sample Diluent 1 (diluted 1:5) into tubes 2 through 7. Add 22.2 µL of the 180 ng/mL 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 Sample Diluent 1 (diluted 1:5) 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 Assay Diluent 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 or 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 Sample Diluent 1 (diluted 1:5).
4. Incubate on ice for 15 minutes. Centrifuge at 2000 x g for 5 minutes, and transfer the supernates into a clean test tube. Assay samples immediately or aliquot and store at ≤ -20 °C in a manual defrost freezer. Sample protein concentration may be quantified using a total protein assay.
5. If needed, further dilute samples in Sample Diluent 1 (diluted 1:5).

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. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
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, prepare the Phospho-HSP27 (S78/S82) Detection Antibody. Add 100 µL of diluted Phospho-HSP27 (S78/S82) 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, prepare the Streptavidin-HRP. Add 100 µL of diluted Streptavidin-HRP to each well. Cover with a new adhesive strip. 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. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, 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.

CALCULATION OF RESULTS

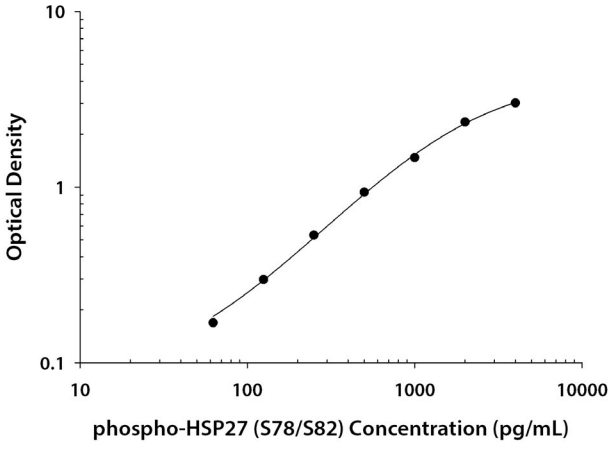
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

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-HSP27 (S78/S82) 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.

If 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-HSP27 (S78/S82) 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	0.040 0.041	—	—
62.5	0.201 0.217	0.161 0.177	0.169
125	0.329 0.347	0.289 0.307	0.298
250	0.560 0.586	0.520 0.546	0.533
500	0.965 0.983	0.925 0.943	0.934
1000	1.411 1.615	1.371 1.575	1.473
2000	2.288 2.484	2.248 2.444	2.346
4000	2.957 3.152	2.917 3.112	3.015

CALIBRATION

The Human/Mouse/Rat Phospho-HSP27 (S78/S82) Surveyor IC Immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-HSP27 (S78/S82) produced at R&D Systems. Samples containing natural phospho-HSP27 (S78/S82) showed linear dilution parallel to the standard curve obtained using the Phospho-HSP27 (S78/S82) Standard. These results indicate that O.D. values from this Surveyor IC Immunoassay can be used to determine the concentration of phospho-HSP27 (S78/S82) in natural samples.

SPECIFICITY

The Human/Mouse/Rat Phospho-HSP27 (S78/S82) Surveyor IC Immunoassay specifically recognizes HSP27 dually phosphorylated at S78 and S82. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody coated on the plate.

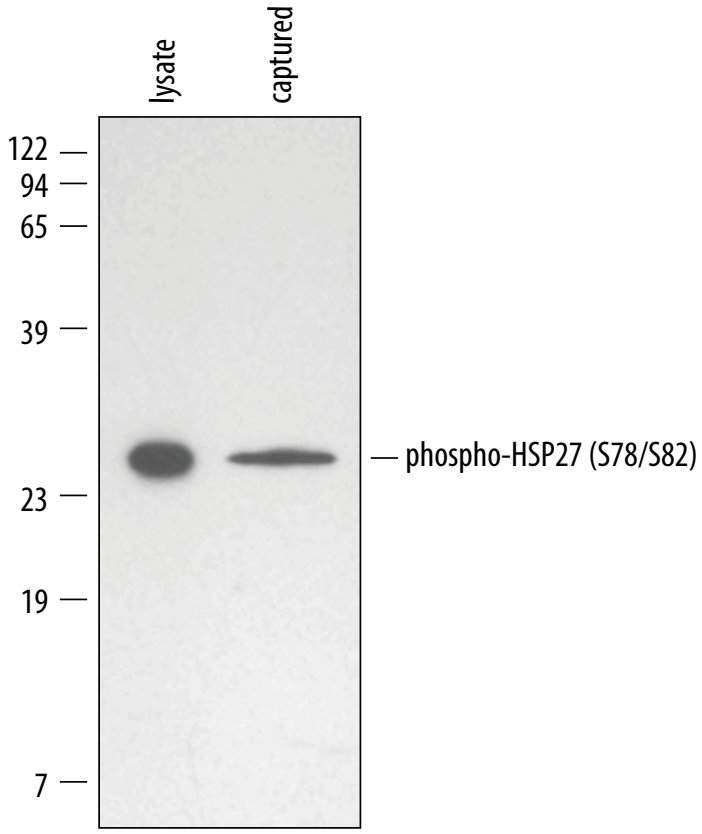


Figure 1: Lysates prepared from HeLa human cervical epithelial carcinoma cells treated with UV (200 J/m^2) were incubated in wells coated with Phospho-HSP27 (S78/S82) Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were electrophoresed, transferred to a PVDF membrane, and immunoblotted with Phospho-HSP27 (S78/S82) Detection Antibody. Only one band corresponding to HSP27 phosphorylated at S78/S82 was detected in the captured material.

To further determine specificity, unphosphorylated recombinant human HSP27 was assayed at 100 ng/mL and exhibited no cross-reactivity or interference in this assay. Unphosphorylated recombinant human HSP60 and HSP70 were also assayed at 100 ng/mL and exhibited no cross-reactivity or interference in the assay.

QUANTIFICATION

Amounts of human phosphorylated HSP27 (S78/S82), as quantified by the Human/Mouse/Rat Phospho-HSP27 (S78/S82) Surveyor IC ELISA, are consistent with the relative amounts of phosphorylated HSP27 determined by qualitative Western blot analysis.

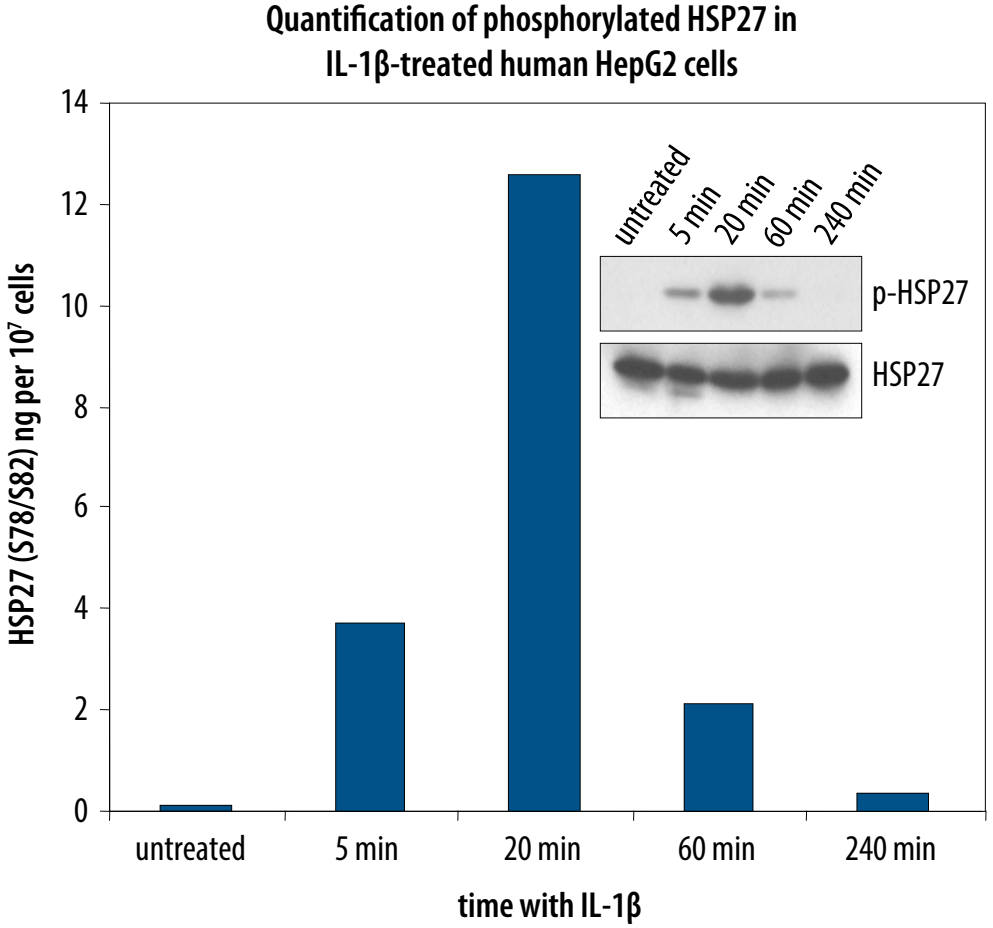


Figure 2: Lysates prepared from HepG2 human hepatocellular carcinoma cells induced with 10 ng/mL of recombinant human IL-1β (R&D Systems, Catalog # 201-LB) for the indicated times were quantified with this Surveyor IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-HSP27 (S78/S82) (p-HSP27) (R&D Systems, Catalog # AF2314) or anti-HSP27 (R&D Systems, Catalog # AF15801) polyclonal antibodies. The Surveyor IC ELISA results correlate well with the relative amounts of phosphorylated HSP27 detected by Western blot. The immunoblot with anti-HSP27 antibody indicates that total levels of HSP27 remained constant during the inductions with recombinant human IL-1β.

QUANTIFICATION *CONTINUED*

The quantification of phosphorylated HSP27 (S78/S82) with this Surveyor IC ELISA was also determined using cells pretreated with the selective p38 MAP kinase inhibitor SB202190 (Tocris, Catalog # 1264), which indirectly blocks the phosphorylation of HSP27.

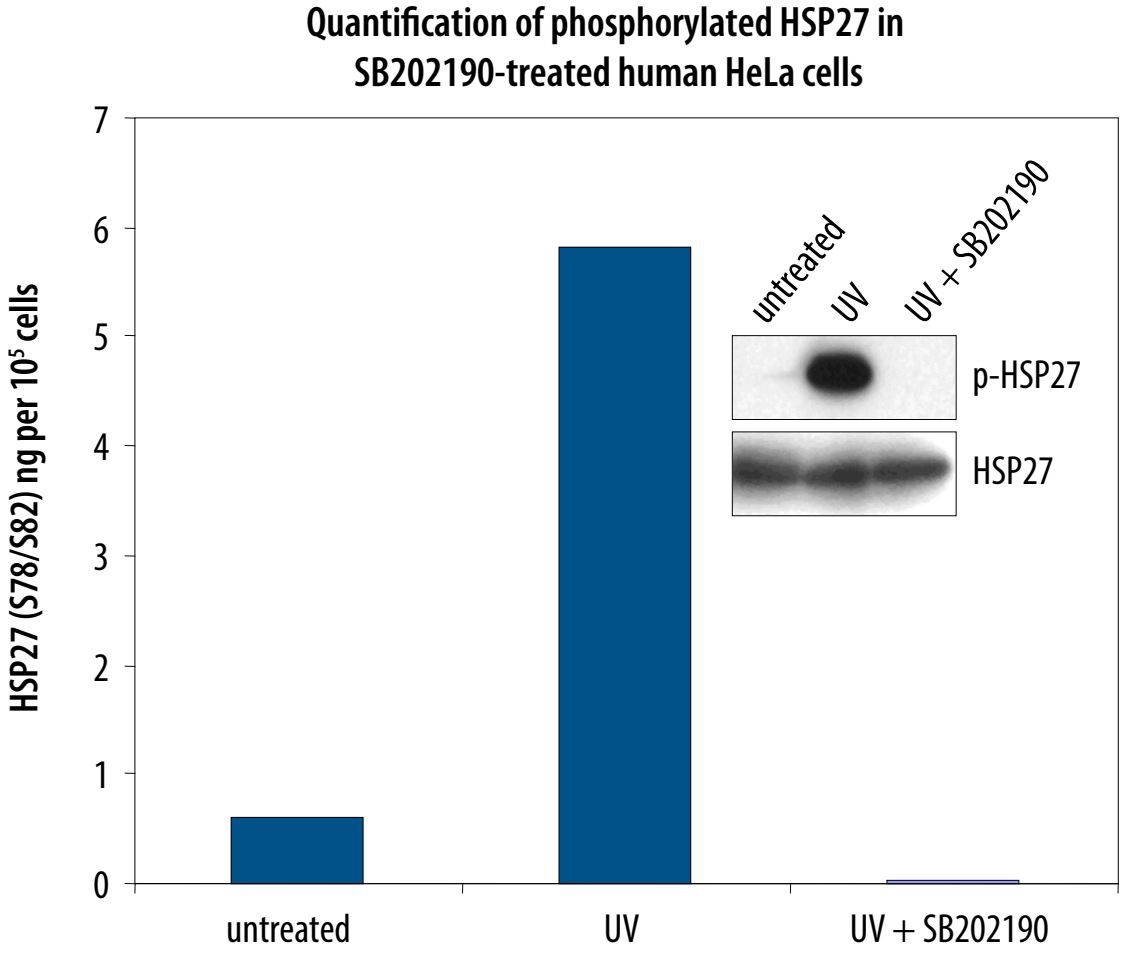


Figure 3: HeLa human cervical epithelial carcinoma cells were incubated with no treatment or were treated with UV (200 J/m^2), either with or without 10 µM SB202190. Cells were lysed and phosphorylated HSP27 was quantified with this Surveyor IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-HSP27 (S78/S82) (p-HSP27) or anti-HSP27 polyclonal antibodies. The Surveyor IC ELISA results correlate well with the relative amounts of phosphorylated HSP27 detected by Western blot. The immunoblot with anti-HSP27 antibody indicates that total levels of HSP27 remained constant during the various treatments.

QUANTIFICATION *CONTINUED*

This DuoSet IC ELISA also quantifies phosphorylated HSP27 levels in mouse and rat cell lysates.

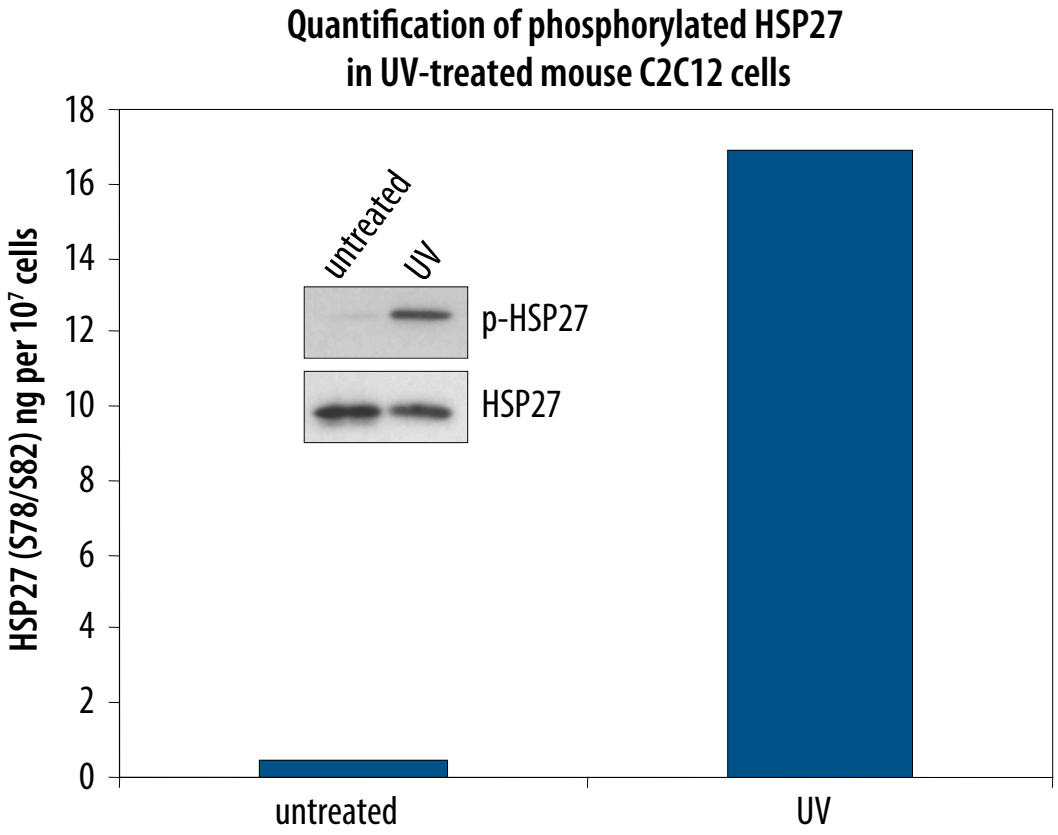


Figure 4: Lysates prepared from C2C12 mouse myoblast cells either untreated or treated with UV (200 J/m^2) were quantified with this Surveyor IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-HSP27 (S78/S82) (p-HSP27) or anti-HSP27 polyclonal antibodies. The Surveyor IC ELISA results correlate well with the relative amounts of phosphorylated HSP27 detected by Western blot. The immunoblot with anti-HSP27 antibody indicates that total levels of HSP27 remained constant during the UV treatment.

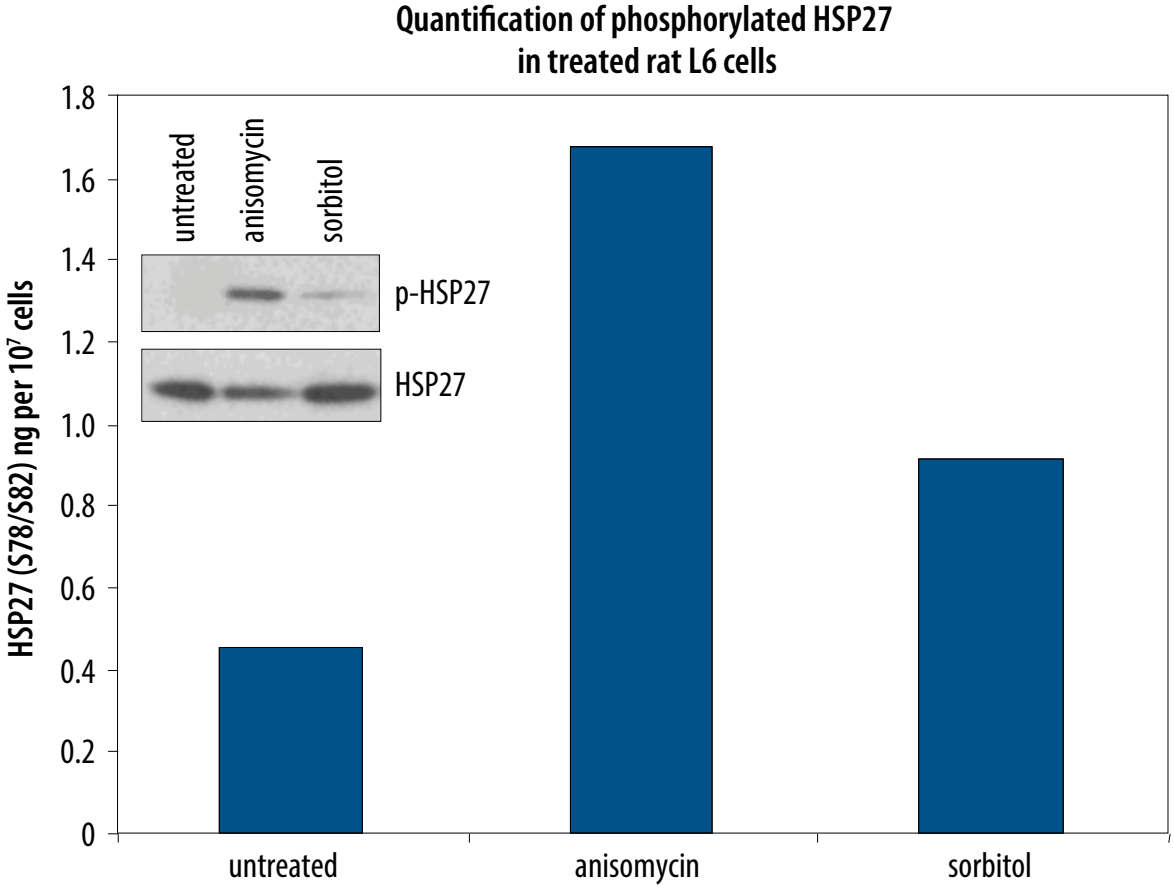


Figure 5: Lysates prepared from L6 rat myoblast cells either untreated or treated with 25 µg/mL anisomycin or 300 mM sorbitol for 20 minutes were quantified with this Surveyor IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-HSP27 (S78/S82) (p-HSP27) or anti-HSP27 polyclonal antibodies. The Surveyor IC ELISA results correlate well with the relative amounts of phosphorylated HSP27 detected by Western blot. The immunoblot with anti-HSP27 antibody indicates that total levels of HSP27 remained constant during treatments.

REFERENCES

1. Arya, R. *et al.* (2007) J. Biosci. **32**:595.
2. Landry, J. *et al.* (1989) J. Cell Biol. **109**:7.
3. Jakob, U. *et al.* (1993) J. Biol. Chem. **268**:1517.
4. Miron, T. *et al.* (1991) J. Cell Biol. **114**:255.
5. Lasa, M. *et al.* (2000) Mol. Cell. Biol. **20**:4265.
6. Mehlen, P. *et al.* (1996) J. Biol. Chem. **271**:16510.
7. Frohli, E. *et al.* (1993) Gene **128**:273.
8. Stokoe, D. *et al.* (1992) FEBS Lett. **313**:307.
9. Landry, J. *et al.* (1991) J. Cell. Physiol. **147**:93.
10. Huot, J. *et al.* (1996) Cancer Res. **56**:273.
11. New, L. *et al.* (1998) EMBO J. **17**:3372.
12. Butt, E. *et al.* (2001) J. Biol. Chem. **276**:7108.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.