DuoSet® IC

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

Catalog Number DYC2314-2
DYC2314-5

For the development of sandwich ELISAs to measure Heat Shock Protein 27 (HSP27) phosphorylated at S78 and 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.

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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 Heat Shock Protein 27 (HSP27) phosphorylated at S78 and S82 in cell lysates. An immobilized capture antibody specific for human/mouse/rat HSP27 (also known as HSPB1 and stress-responsive protein 27) binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody that recognizes 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.

MATERIALS PROVIDED

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

			Vials Provided	
Description	**Part #**	**Storage Conditions**	**Cat. # DYC2314-2**	**Cat. # DYC2314-5**
Human/Mouse/Rat Phospho-HSP27 (S78/S82) Capture Antibody	841757	2-8 °C	1	2
Human/Mouse/Rat Phospho-HSP27 (S78/S82) Detection Antibody	841758	2-8 °C	1	2
Human/Mouse/Rat Phospho-HSP27 (S78/S82) Standard	841759	2-8 °C	3	5
Streptavidin-HRP	890803	2-8 °C	1	1

DYC2314-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC2314-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

*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)
- Triton™ X-100 (Sigma # T9284)
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates (R&D Systems' Catalog # DY990)
- 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 (R&D Systems, Catalog # DY006).

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.

IC Diluent #4** - 1 mM EDTA, 0.5% Triton X-100 in PBS, pH 7.2-7.4.

Note: *Approximately 50 mL of this diluent is required to run the assay on one plate.*

Lysis Buffer #12** - 1 mM EDTA, 0.5% Triton X-100, 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 (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.

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REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human/Mouse/Rat Phospho-HSP27 (S78/S82) Capture Antibody (Part 841757) - Each vials contains 360 μg/mL of goat anti-human HSP27 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.*

Human/Mouse/Rat Phospho-HSP27 (S78/S82) Detection Antibody (Part 841758) - Each vials contains 18 μg/mL of biotinylated rabbit anti-human phospho-HSP27 (S78/S82) 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.*

Human/Mouse/Rat Phospho-HSP27 (S78/S82) Standard (Part 841759) - Each vials contains 170 ng/mL of recombinant human phospho-HSP27 (S78/S82) when reconstituted with 500 μL of IC Diluent #4. **Use within one hour of reconstitution. A fresh standard should be used for each assay.** A seven point standard curve using 2-fold serial dilutions and a high standard of 4000 pg/mL is recommmended.

Streptavidin-HRP (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using IC Diluent #1. 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 #12 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. If needed, further dilutions should be made in IC Diluent #4.

PRECAUTIONS

The Stop Solution recommended for use with this kit is an acid solution.

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

Color Reagent B recommended for use with this kit 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.

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 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 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 #4 per well. Use IC Diluent #4 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 4000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Immediately before use, dilute the Detection Antibody to a working concentration of 500 ng/mL in IC Diluent #1. Prepare only as much Detection Antibody as required to run each assay. 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. 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.

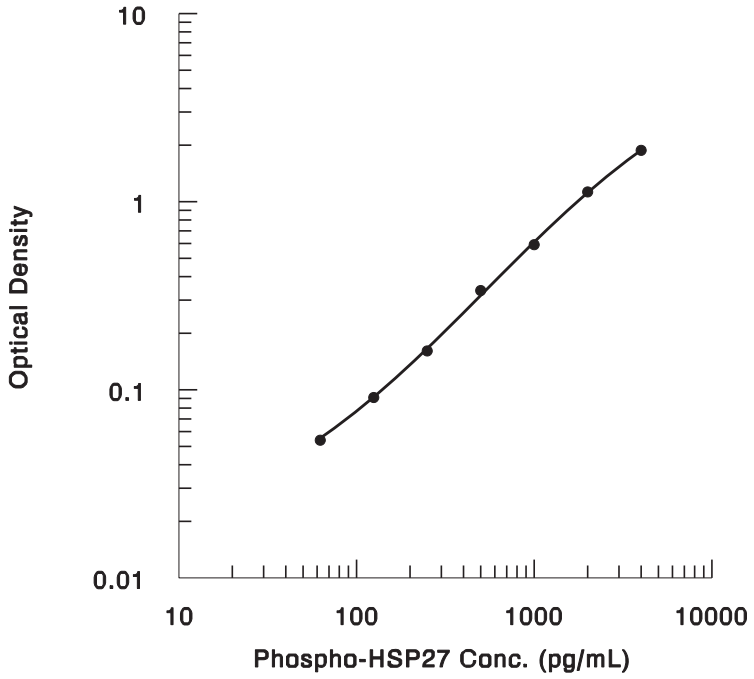
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample then subtract the average zero standard optical density (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 Huamn/Mouse/Rat 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.

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) 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-HSP27 (S78/S82) DuoSet® IC ELISA 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 DuoSet® IC ELISA can be used to determine the relative concentration of phospho-HSP27 (S78/S82) in natural samples.

SPECIFICITY

The Human/Mouse/Rat Phospho-HSP27 (S78/S82) DuoSet® IC ELISA specifically recognizes phosphorylated HSP27. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody supplied in the ELISA.

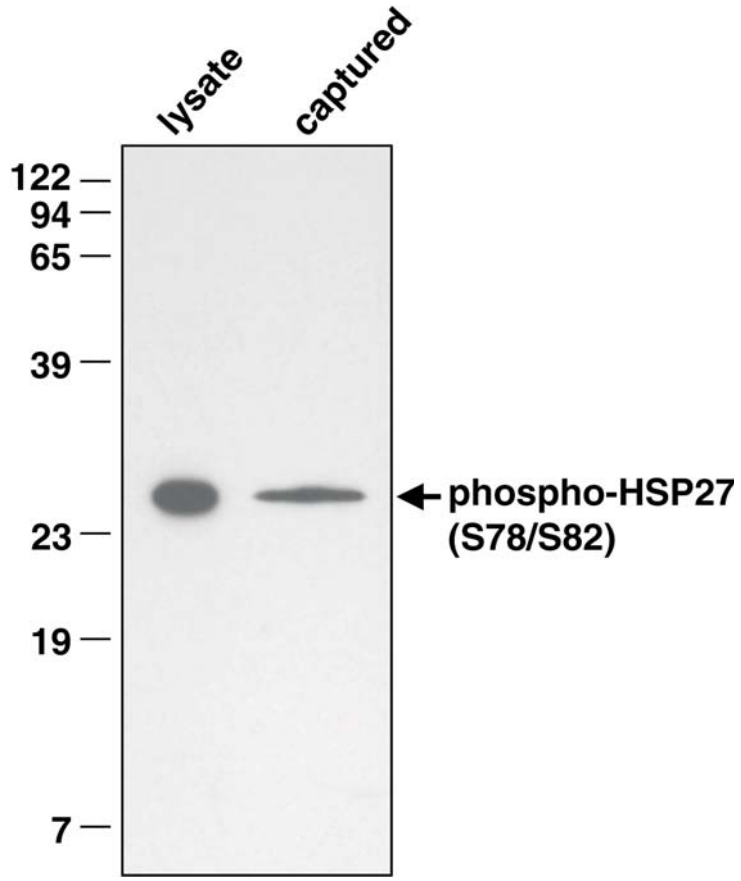


Figure 1: Lysates prepared from HeLa human cervical epithelial carcinoma cells treated with UV (200 J/m^2) were incubated in wells coated with Human /Mouse/Rat Phospho-HSP27 (S78/S82) Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. 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 captured material.

To further determine specificity, recombinant human HSP27 was assayed at 100 ng/mL and exhibited no cross-reactivity or interference. Recombinant human (rh) HSP60 and rhHSP70 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) DuoSet® IC ELISA, are consistent with the amounts of phosphorylated HSP27 determined by qualitative Western blot analysis.

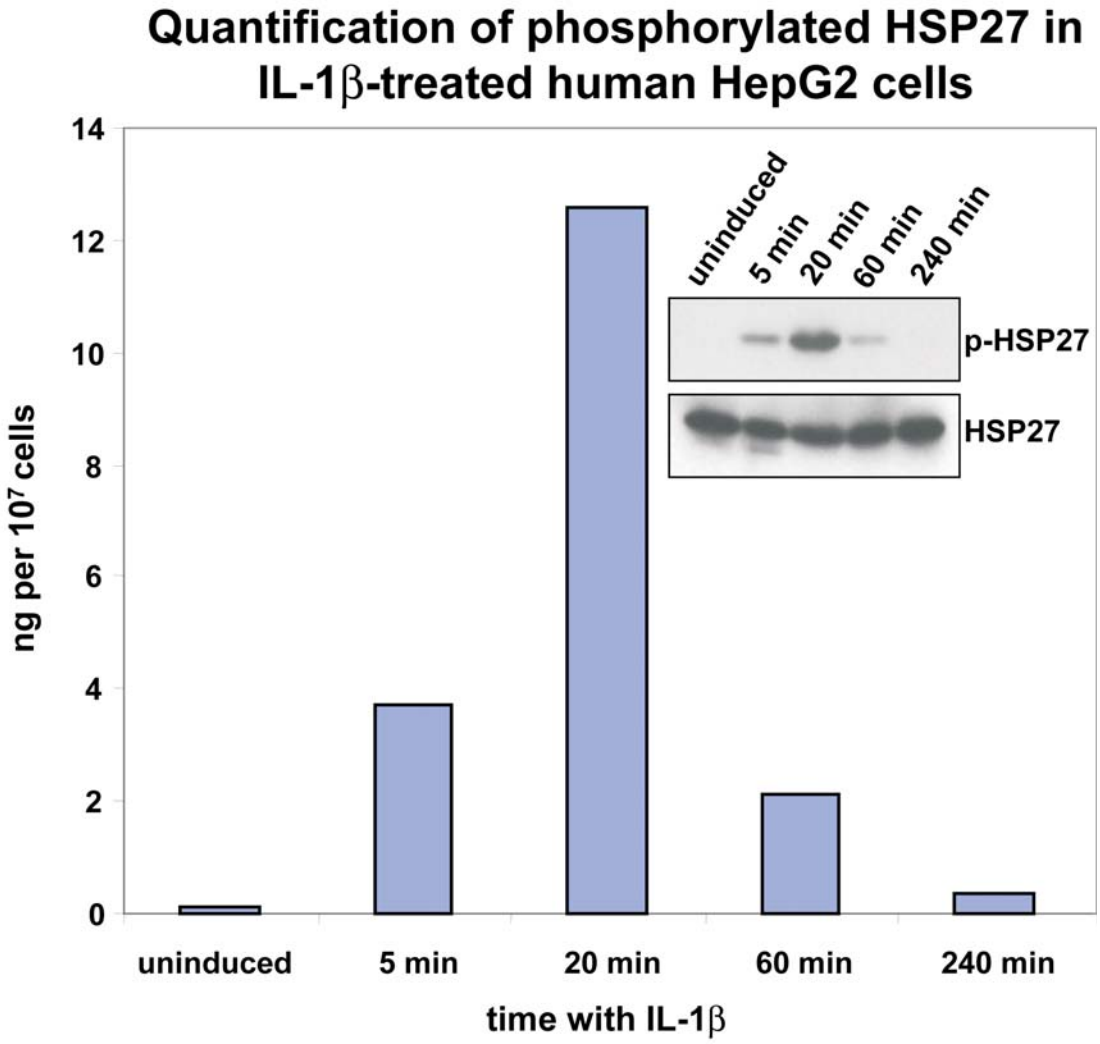


Figure 2: Lysates prepared from HepG2 human hepatocellular carcinoma cells induced with 10 ng/mL of human IL-1β (R&D Systems, Catalog # 201-LB) for the indicated times were quantified with this DuoSet® IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-HSP27 (S78/S82) (p-HSP27) (R&D Systems, Catalog # AF2314) or anti-HSP27 (R&D Systems, Catalog # AF15801) polyclonal antibodies. The DuoSet® IC ELISA results correlate well with the 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 human IL-1β.

The quantification of phosphorylated HSP27 (S78/S82) with this DuoSet® 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.

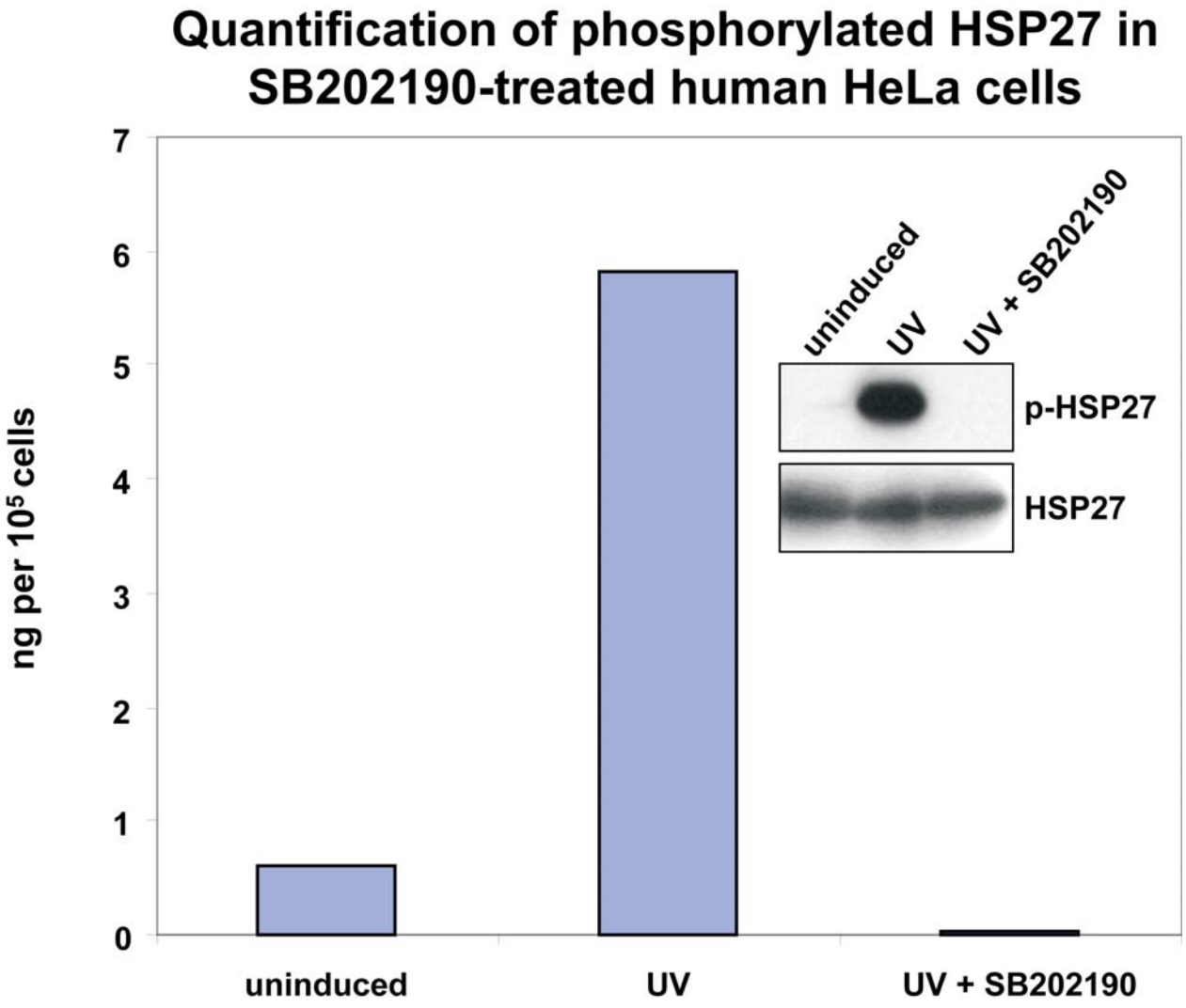


Figure 3: HeLa human cervical epithelial carcinoma cells were incubated with no treatment or were treated with UV (200 J/m^2), either with or without 10 μM SB202190. Cells were lysed and phosphorylated HSP27 was quantified with this DuoSet® IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-HSP27 (S78/S82) (p-HSP27) or anti-HSP27 polyclonal antibodies. The DuoSet® IC ELISA results correlate well with the 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.

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

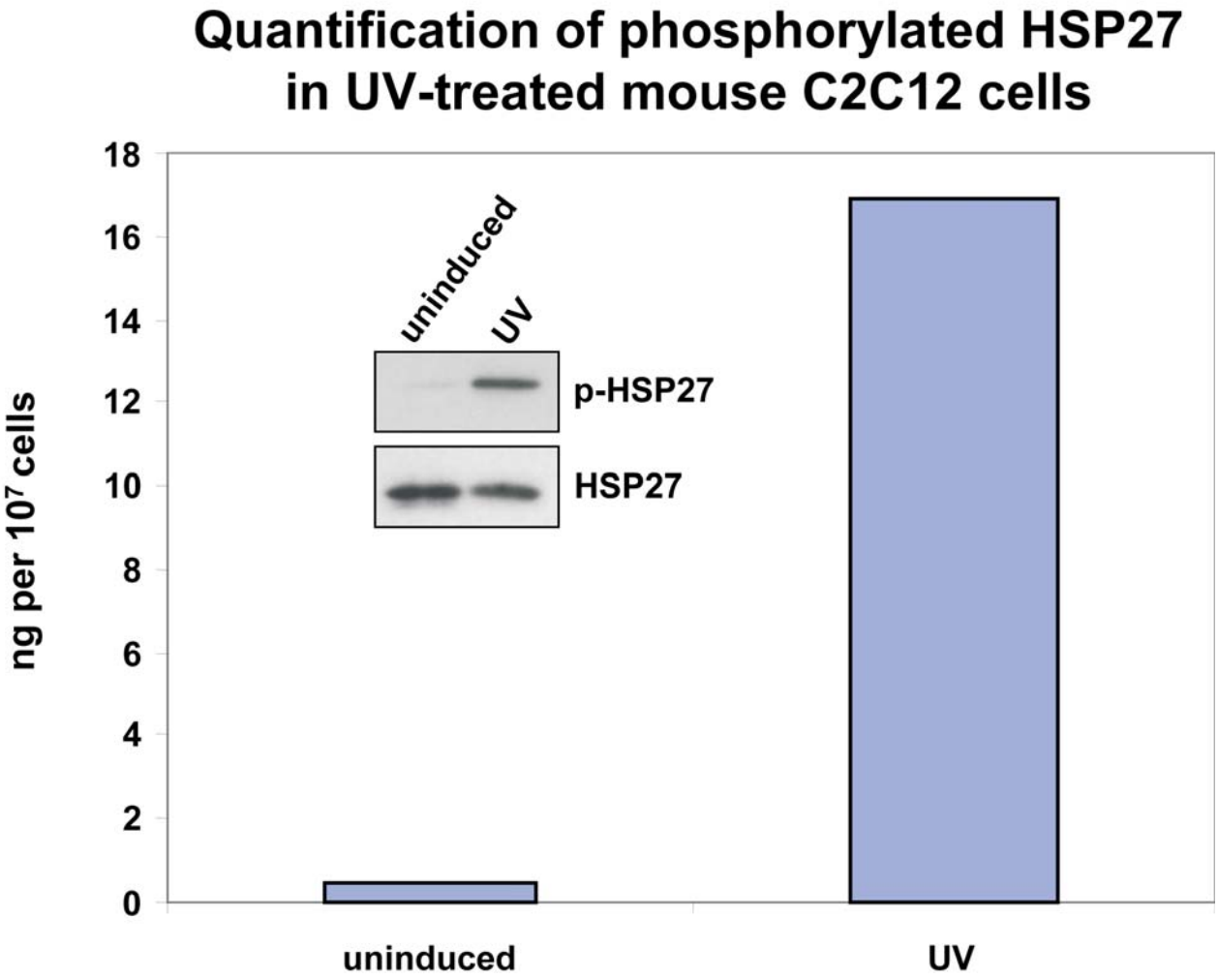


Figure 4: Lysates prepared from C2C12 mouse myoblast cells either uninduced or treated with UV (200 J/m^2) were quantified with this DuoSet® IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-HSP27 (S78/S82) (p-HSP27) or anti-HSP27 polyclonal antibodies. The DuoSet® IC ELISA results correlate well with the 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 uninduced or treated with anisomycin (25 μg/mL) or sorbitol (300 mM) for 20 minutes were quantified with this DuoSet® IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-HSP27 (S78/S82) (p-HSP27) or anti-HSP27 polyclonal antibodies. The DuoSet® IC ELISA results correlate well with the amounts of phosphorylated HSP27 detected by Western blot. The immunoblot with anti-HSP27 antibody indicates that total levels of HSP27 remained constant during treatments.