3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

Heat Shock Protein 27 (HSP27) ELISA, Human

Product Number **CS0640**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description
Heat Shock Protein 27 ELISA is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). for the quantitative determination of Heat Shock Protein 27 (HSP27). A monoclonal antibody specific for HSP27 (regardless of phosphorylation state) has been coated onto the wells of the multiwell plate strips provided. HSP27 standard dilutions, control specimens, unknown samples and Anti-HSP27 detection antibody are pipetted into these wells. During the first incubation, the HSP27 antigen binds simultaneously to the immobilized (capture) antibody and to the solution phase detection antibody specific for total HSP27. After washing, a horseradish peroxidase-labeled anti-rabbit IgG (anti-rabbit IgG-HRP) is added. This binds to the detection antibody to complete the four-member sandwich. After the second incubation and washing to remove excess anti-rabbit IgG-HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of HSP27 present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of HSP27.

Heat Shock Protein 27 ELISA is designed to detect and quantify the level of HSP27, independent of its phosphorylation state. This assay is intended for the detection of HSP27 from lysates of human cells. It does not react with the mouse or rat HSP27 homolog, HSP25. It can be used to normalize the HSP27 content of the samples when examining quantities of phosphorylated sites on HSP27 using Sigma Phospho-Heat Shock Protein [pSer^{82}] ELISA (Product No. CS0630).

HSP27 (Estrogen-regulated 24 kDa protein) is a member of the ubiquitously expressed anti-apoptotic heat shock protein group with MW of 27 kDa. Other members of this group include HSP70 and HSP90. Human HSP27 shares 80% homology with the mouse homolog HSP25 and 43% homology with the *C. elegans* protein C09B8.6. HSP27's N-terminal region contains several phosphorylatable serine residues (serine 15, serine 78, and serine 82 [serine 85 in the mouse]). Other regions of HSP27 include a conserved α-crystallin domain and a flexible C-terminal domain. The protein is diffusely distributed throughout the cytoplasm, and is also found associated with the mitochondrial outer membrane. HSP27 occurs as monomers, dimers, and homotypic oligomers, as well as in complexes with other proteins, suggesting a role for HSP27 as a scaffolding protein and as a molecular chaperone. Recently, HSP27 has been observed to be an important part of a signaling module that also contains Akt, p38, and MAPKAPK-2, where it is found to modulate the activity of Akt.

HSP27 expression is regulated at the level of transcription and translation in response to diverse stimuli, including heat shock, oxidative stress, IL-1, TNF-α, chemotherapeutic agents, staurosporine, Fas ligand, cadmium, and estrogen in estrogen-responsive cells. Transcription factors implicated in HSP27 expression include Hsf-1, as well as STAT1, ATF3, and c-Jun. Heat shock induces HSP27 phosphorylation and the dissociation of oligomers. HSP27 phosphorylation is observed with the activation of p38 MAPK and Jnk cascades. In the p38 MAPK cascade, activated p38 MAPK phosphorylates and thereby activates MAPKAPK-2, which in turn phosphorylates HSP27 at serine 82, serine 78, and serine 15. HSP27 is also phosphorylated at serine 82 by Akt. Enzyme activities that dephosphorylate HSP27 include PP2A and PP2B. HSP27 has many functions. HSP27 plays a role in maintaining cellular redox state by increasing glutathione levels, stabilizes actin filaments and blocks actin polymerization, and prevents the aggregation of improperly folded proteins. Recent evidence suggests that HSP27 protects cells from apoptosis by participation in several signaling pathways. By activating Akt within the HSP27/Akt/p38 MAPK/MAPKAPK-2 signaling module, HSP27 inhibits apoptosis through Akt's phosphorylation of BAD, Forkhead Transcription Factor, and caspase-9. By

binding to cytochrome c, mediated by amino acids 51-88 of the α-crystalline domain, HSP27 prevents the formation of the apoptosome and activation of caspase cascades. By binding to the adaptor protein Daxx, HSP27 prevents apoptosis by inhibiting the association of Daxx with Fas and Ask-1. HSP27 also acts to prevent apoptosis by activating NF-κB-dependent gene transcription through enhancing the rate of degradation of ubiquinated IκBα by the 26S proteasome.

HSP27 is under intensive investigation in several areas of research, including cancer studies and neurology. HSP27 is overexpressed in many tumors. HSP27 expression levels are correlated with oncogenicity and resistance to chemotherapeutics. HSP27 is upregulated in many gynecological cancers and can be detected extracellularly complexed with cytochrome c. Detection of these extracellular complexes may provide a facile screening method. HSP27 protects neurons from numerous stresses, including ischemia, axotomy, NGF withdrawal, kainic acid treatment, retinoic acid treatment, and thermal stress. HSP27 is also of interest in normal tissue homeostasis, as this protein may play a role in protecting cells from apoptosis in response to growth cessation as they enter the G1 phase.

Reagents

- **HSP27 Standard, Lyophilized, 2 vials, Product No. H 6413-** Full length recombinant HSP27 expressed in *E. coli,* Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 3068,** contains sodium azide as preservative.
- **Monoclonal-Anti-HSP27-Coated 96 well plate, 1EA, Product No. H 6163 -** A plate using break-apart strips coated with monoclonal antibody specific for full-length HSP27 (regardless of phosphorylation state).
- **Anti-HSP27, 6 mL, Product No. H 6038** A detection antibody, produced in rabbit. Contains sodium azide. Ready to use.
- **Anti-Rabbit IgG-HRP, Concentrate (100X), 1 vial, Product No. R 8278 -** contains 3.3 mM thymol and 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 5788 -** contains 3.3 mM thymol. Ready to use.
- **Wash Buffer concentrate, 25X, 100 mL, Product No. W 2639 -** *See Reagent Preparation for handling, dilution and storage instructions*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm.
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL.
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.

Cell Extraction Buffer

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM Na_3VO_4
1% Triton® X-100
10% glycerol
0.1% SDS
0.5% deoxycholate
1 mM PMSF (stock is 0.3 M in DMSO) *PMSF is very unstable and must be added prior to use, even if added previously*.
Protease inhibitor cocktail (Sigma Product No. P 2714)

Add 250 µl of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 °C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 °C.

Thaw on ice. *Add the protease inhibitors just before use.*

Procedure for Extraction of Proteins from Cells

This protocol has been successfully applied to several cell lines of human origin. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at –70 °C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals.
5. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of HSP27. For example, $1X10^6$ THP1 cells grown in DMEM plus 10% FBS can be extracted in 1 mL of Cell Extraction Buffer. Under these conditions, use of 1-5 µL of the clarified cell extract diluted to a volume of 50 µL/well in Standard Diluent Buffer is sufficient for the detection of HSP27.
6. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 °C.
7. Aliquot the clear lysate to clean microcentrifuge tubes

Before assay: extracted cell lysate samples containing protein should be diluted with Standard Diluent Buffer at least 1:10. This dilution is necessary to reduce the matrix effect of the cell extraction buffer.

Reagent Preparation

Standard

Note: This HSP27 standard was prepared from full length, recombinant HSP27 protein.

1. Reconstitute HSP27 Standard with *Standard Diluent Buffer*. Refer to standard vial label for instructions.
2. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Label as 50 ng/mL Use standard within 1 hour of reconstitution.
3. Prepare serial standard dilutions as follows

Tube#	Standard Buffer	Standard from tube #:	Final ng/mL
1	Reconstitute according to label instructions		50 ng/mL
2	0.15 mL	0.15 mL (1)	25 ng/mL
3	0.15 mL	0.15 mL (2)	12.5 ng/mL
4	0.15 mL	0.15 mL (3)	6.25 ng/mL
5	0.15 mL	0.15 mL (4)	3.12 ng/mL
6	0.15 mL	0.15 mL (5)	1.6 ng/mL
7	0.15 mL	0.15 mL (6)	0.8 ng/mL
8	0.15 mL		0 ng/mL

4. Remaining reconstituted standard should be discarded or frozen at –70 °C. for further use. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Anti-rabbit IgG Horseradish Peroxidase (HRP)

Note: The *Anti-rabbit IgG-HRP* 100X concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution:

1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Within 1 hour of use, dilute 10 µL of this 100X concentrated solution with 1 mL of *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-rabbit IgG-HRP Working Solution.

4. Return the unused concentrate to the refrigerator
5. For more strips use the following amounts:

# of 8 well strips	IgG-HRP Concentrate µL	Diluent mL
2	20	2
4	40	4
6	60	6
8	80	8
10	100	10
12	120	12

Wash Buffer

1. Equilibrate to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate 25X + 24 volumes of deionized water
3. Label as *Working Wash Buffer.*
4. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days.

Storage/Stability

All components of this kit are stable at 2-8 °C. Any unused reconstituted standard should be discarded or frozen at –70 °C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www.sigma-aldrich.com

Procedure

Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 °C).
- Use only the coated 96 well capture plate provided with the kit
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2-8 °C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of component and reagent addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle four times, blotting as dry as possible after the 4th wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

HSP27 ELISA Assay Summary

1) 50 µL of HSP27 Standards or Samples (samples diluted 1:10 or higher in Standard Diluent Buffer) and 50 µL Anti-HSP27
Incubate 2 hours at RT
aspirate and wash 4x

2) Add 100 µL Anti-Rabbit IgG-HRP
Incubate 30 min at RT.
aspirate and wash 4x

3) Add 100 µL Stabilized Chromogen
Incubate 30 minutes at RT
(in the dark).

4) Add 100 µL of Stop Solution
Read at 450nm.

Total Assay Time - 3 hours

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilution wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch

1st incubation

a Add 50 µL Standard Diluent to zero wells.
b Add 50 µL standards, samples or controls to the appropriate wells.
c Samples prepared in cell extraction buffer must be diluted 1:10 or greater in *Standard Diluent Buffer* (for example, 10 µL sample plus 90 µL buffer). *The dilutions should be optimized for each assay.*
d Cell culture supernatants or buffered solutions; dilute 1:2 in *Standard Diluent Buffer* (50 µL buffer + 50 µL sample).
e Add 50 µL Anti-HSP27 detection antibody to all wells (except chromogen blanks).
f Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature.
g Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells for a total of 4 times following washing instructions

2nd incubation

a Add 100 µL Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
b Cover with Plate Cover and incubate 30 minutes at room temperature.
c Wash wells for a total of 4 times following washing instructions.
d After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

a Add 100 µL of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*
b Do not cover the plate
c Incubate approximately 30 minutes at room temperature in the dark (*place plate in a drawer or cabinet).*

Note: *If your multiwell plate reader does not register optical density (OD) above 2.0, incubate only 20-25 minutes.*

Stop reaction

a Add 100 µL of Stop Solution to each well. This stops the reaction
b Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

a Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
b Blank the plate reader against the Chromogen Blank wells (contain Chromogen and Stop Solution).

Read the absorbance of the entire plate at 450 nm within 2 hours after addition of Stop Solution

Results

1. The results may be calculated using any immunoassay software package.
2. The four-parameter algorithm provides the best curve fit.
3. If the software program is not readily available, the concentrations of HSP27 may be calculated manually.
4. Calculate the Average Net OD (average reading of 2 wells) for each standard dilution and samples as follows:
5. Average Net OD = Average Bound OD– Average Chromogen Blank OD
6. On graph paper plot the Average Net OD of standard dilutions against the standard concentration (ng/mL) of HSP27. Draw the best curve through these points to construct the standard curve.
7. The HSP27 concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
8. Multiply the values obtained for the samples by dilution factor of each sample.
9. Samples producing signals higher than the 50 ng/mL standard should be further diluted and assayed again.

Product Profile

Typical Results

The standard curve below is for illustration only and **should not be used** to calculate results in your assay. Run standard curve in each assay.

Standard Curve

Standard HSP27 (ng/mL)	Optical density (450 nm)
50.0	3.27
25.0	2.37
12.5	1.59
6.25	1.06
3.12	0.71
1.6	0.48
0.8	0.34
0	0.19

Limitations

- Do not extrapolate the standard curve beyond the 50 ng/mL standard point.
- The dose response is non-linear in this region and accuracy will be compromised.
- Other buffers and matrices have not been investigated.
- The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of cell extract and brain homogenate samples have not been thoroughly investigated.
- The rate of degradation of native HSP27 in various matrices has not been investigated.

Performance characteristics

Sensitivity

The analytical sensitivity of this assay is <0.3 ng/mL of HSP27. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. Using THP1 cells. This level of sensitivity was equivalent to the detection of HSP27 in 1000 cells.

The sensitivity of this ELISA was compared to immunoblotting using known quantities of HSP27. The data presented in Figure 1 show that the sensitivity of the ELISA is approximately 4x greater than that of immunoblotting. The bands shown in the immunoblotting data were developed using rabbit anti-HSP27, an alkaline phosphatase conjugated anti-rabbit IgG, followed by chemiluminescent substrate and autoradiography..

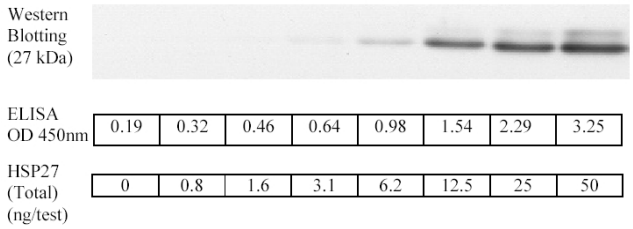

Figure 1 Detection of HSP27 by ELISA vs. immunoblot

Precision

1. Intra-Assay Precision

Samples of known concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	31.5	13.3	3.6
Standard Deviation (SD)	0.8	0.6	0.3
% Coefficient of Variation	2.5	4.5	7.8

2. Inter-Assay Precision

Samples were assayed 42 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	31.9	12.8	3.5
Standard Deviation (SD)	1.2	0.8	0.3
Coefficient of Variation %	3.8	5.9	9.8

Recovery

To evaluate recovery, HSP27 standard was spiked at 3 different concentrations into 10% cell extraction buffer and human plasma. The average recovery in Cell Extraction buffer was 80% while EDTA and citrate plasma recoveries were 84% and 126%, respectively.

Parallelism

Natural HSP27 from THP1cell lysate was serially diluted in *Standard Diluent Buffer.* The optical density of each dilution was plotted against the HSP27 standard curve. Parallelism demonstrated in Figure 2 indicates that the standard accurately reflects HSP27 content in samples.

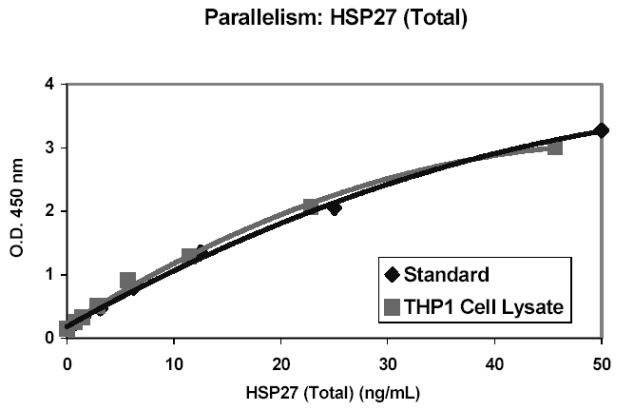

Figure 2 Parallelism: HSP27 total

Linearity of Dilution

THP1 cells were grown in tissue culture medium containing 10% fetal bovine serum and lysed with Cell Extraction Buffer. This lysate was diluted with *Standard Diluent Buffer* over the range of the assay and measured for HSP27 content. Linear regression analysis of sample values versus the expected concentration yielded a correlation coefficient of 0.99.

Dilution	**Cell Lysate**		
	Measured ng/mL	Expected ng/mL	% Expected
Neat	45.6	45.6	100
1:2	25.7	22.8	113
1:4	12.6	11.4	110
1:8	7.4	5.7	130
1:16	3.0	2.9	107
1:32	1.6	1.4	109

Specificity

- The HSP27 ELISA is specific for measurement of total HSP27 protein.
- To determine the specificity of this ELISA , cell extracts from different cell lines, each at a concentration of 50 µg/mL total protein, were analyzed.
- The data presented in Figure 3 show that the kit detects HSP27 protein in cell lysates from human HTP1, HeLa and 293 cells. It detects low levels in human HT1080 and Jurkat cells.
- The levels of HSP27 protein detected with this ELISA kit are consistent with results obtained by immunoblot analysis (insert).

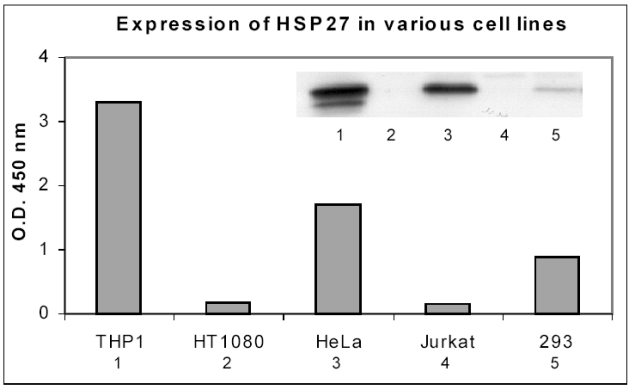

Figure 3 Expression of HSP27 in various cell lines

References

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