Product Manual

[pSer^{78}]HSP27 (human), ELISA kit

Catalog #: ADI-900-165

96 well Enzyme-Linked ImmunoSorbent assay kit

For use with cell lysates, serum and plasma

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Product Manual

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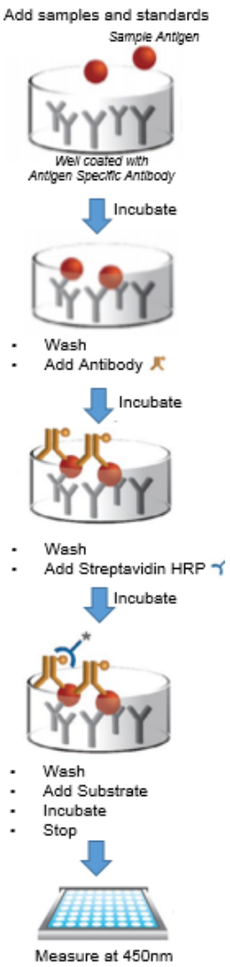
Carefully note the handling and storage conditions of each kit component.

INTRODUCTION

Hsp27 is one of the most common members of the highly conserved and ubiquitously expressed family of small heat shock proteins (sHsp), which also includes αB-crystallin[1]. It is characterized by a conserved C-terminal α-crystallin domain consisting of two anti-parallel β-sheets that promote oligomer formation required for its primary chaperone function as an inhibitor of irreversible protein aggregation[2]. Hsp27 oligomerization is modulated by post-translational phosphorylation of Hsp27 at three serine residues, Ser15, Ser78, and Ser82, by a variety of protein kinases including MAPKAPK-3, PKAcα, p70S6K, PKD I, and PKCδ[3, 4]. Hsp27 has been shown to inhibit actin polymerization by binding of unphosphorylated Hsp27 monomers to actin intermediate filaments[5]. Anti-apoptotic functions of Hsp27 have also been identified through interactions with DAXX7, activation of Akt, and inhibition of apoptosome formation[6-8]. Evidence suggests altered expression of Hsp27 is implicated in the pathogenesis of breast, ovarian, and prostate cancer[9].

PRINCIPLE OF THE ASSAY



1. Samples and standards are added to wells coated with a monoclonal antibody specific for Hsp27. The plate is then incubated.

2. The plate is washed, leaving only bound Hsp27 on the plate. A yellow solution of antibody, specific for Hsp27 phosphorylated at Ser78, is then added. This binds the Hsp27 (phospho-Ser78) captured on the plate. The plate is then incubated.

3. The plate is washed to remove excess antibody. A blue solution of HRP conjugate is added to each well, binding to the antibody, which is attached to the Hsp27 (phospho-Ser78). The plate is again incubated.

4. The plate is washed to remove excess HRP conjugate. TMB Substrate solution is added. The substrate generates a blue color when catalyzed by the HRP.

5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of Hsp27 (phospho-Ser78) in the sample.

MATERIALS SUPPLIED

1. Assay Buffer 27
   100 mL, Component number 80-1753
   Phosphate buffered saline containing BSA

2. phospho Hsp27 Standard
   0.25 mL, Component number 80-1676
   One vial containing 10,000 pg/mL of recombinant human phospho Hsp27

3. 5X Extraction Reagent
   10 mL, Component number 80-1526

4. Hsp27 Clear Microtiter Plate
   One Plate of 96 Wells, Component number 80-1560
   A plate of break-apart strips coated with a mouse monoclonal antibody specific for Hsp27

5. Wash Buffer Concentrate
   100 mL, Component number 80-1287
   Tris buffered saline containing detergents

6. Hsp27 (phospho-Ser78) EIA Antibody
   10 mL, Component number 80-1674
   A yellow solution of mouse monoclonal antibody specific for Hsp27, phosphorylated at Ser78

7. Hsp27 (phospho-Ser78) EIA Conjugate
   10 mL, Component number 80-1673
   A blue solution of mouse anti-rabbit IgG conjugated to horseradish peroxidase

Protect substrate from prolonged exposure to light.

8. TMB Substrate
   10 mL, Component number 80-0350
   A solution of 3,3',5,5' tetramethylbenzidine (TMB) and hydrogen peroxide

Stop solution is caustic. Keep tightly capped.

9. Stop Solution 2
   10 mL, Component number 80-0377
   A 1N solution of hydrochloric acid in water

10. Hsp27 (phospho-Ser78) Assay Layout Sheet
    1 each, Component number 30-0244

11. Plate Sealer
    3 each, Component number 30-0012


Storage temp

STORAGE

All components of this kit, except the standard, are stable at 4ºC until the kit's expiration date. The standard must be stored at or below -20ºC.

ADDITIONAL MATERIALS NEEDED

- Deionized or distilled water
- Phenylmethylsulfonyl fluoride (PMSF), Sigma #P7626 or equivalent
- Protease inhibitor cocktail (PIC), Sigma #P8340 or equivalent
- Phosphatase inhibitor cocktail (PhIC), Sigma #P2850 or equivalent
- Precision pipets for volumes between 5 µL and 1,000 µL
- Repeater pipet for dispensing 100 µL
- Disposable beakers for diluting buffer concentrates
- Graduated cylinders
- A microplate shaker
- Lint-free paper for blotting
- Microplate reader capable of reading at 450 nm
- Graph paper for plotting the standard curve

REAGENT PREPARATION

1. **Wash Buffer**

   Prepare the wash buffer by diluting 50 mL of the supplied Wash Buffer Concentrate with 950 mL of deionized water. This can be stored at room temperature until the kit's expiration, or for 3 months, whichever is earlier.

2. **Extraction Reagent**

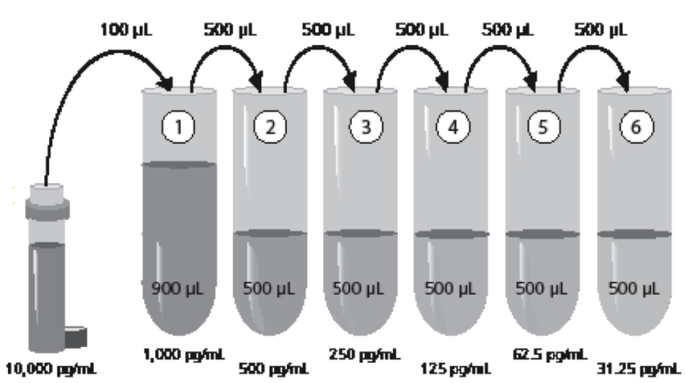
   Prepare the Extraction Reagent by diluting 10 mL of the supplied 5X Extraction Reagent with 45 mL of deionized water. This can be stored at 4°C until the kit expiration, or for 3 months, whichever is earlier.

3. **PIC, PhIC, and PMSF Addition**

   Immediately prior to use; PIC, PhIC, and PMSF must be added to the Extraction Reagent. If using Sigma Protease Inhibitor Cocktail #P8340, add 0.5 µL/mL PIC, or equivalent concentration according to alternate vendor's specification sheet. If using Phosphatase Inhibitor Cocktail #P2850, add 10 µL/mL PhIC, or equivalent concentration according to alternate vendor's specification sheet. Add PMSF, such as Sigma #P7626, to a final concentration of 1 mM.

   Inhibitors must be freshly added to the Extraction Reagent to ensure optimal integrity of the samples. Each inhibitor treated buffer should incubate for 5-10 minutes at room temperature before it is used. Buffers treated with inhibitors should be used within 1 hour of preparation.

4. **Preparation of phospho Hsp27 Standard Curve**



Label six 12x75 mm polypropylene tubes #1 through #6. Pipet 900 µL of the assay buffer into tube #1. Pipet 500 µL of the assay buffer into tubes #2 through #6. Add 100 µL of the 10,000 pg/mL standard into tube #1. Vortex thoroughly. Add 500 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 though #6.

Diluted standards should be used within 1 hour of preparation. The concentrations of the standards are labeled above.

SAMPLE HANDLING

This assay is suitable for measuring Hsp27 phosphorylated at Ser78 in cell lysates, serum, and plasma. Prior to assay, frozen samples should be slowly brought to 4ºC and centrifuged, if necessary, to isolate residual debris.

For cell lysates, a minimum 1:2 dilution of the 1X extraction reagent into the assay buffer is required to remove matrix interference of this buffer. Due to differences in sample types, number of cells, or total cellular protein concentration, samples may require greater dilution with the assay buffer to remove interference or to be read within the standard range. Users must determine the optimal sample dilutions for their particular experiments. Below are examples of sample recoveries with several different sample types. Note that % recovery was calculated based on linearity of samples.

Sample	Total cellular protein (mg/mL)	% Recovery	Recommended Dilution
HeLa cells (human)	1.55	89%	1:50
Serum (human)	N/A	99%	1:2
EDTA Plasma (human)	N/A	99%	1:100

Cell Lysate Preparation

1. Harvest cells and centrifuge at 250 x g (~1534 rpm) for 7 minutes at 4ºC. Discard supernatant.
2. Resuspend pellet and wash with Hank's Balanced Salt Solution
   (without phenol red) or PBS.
3. Centrifuge at 250 x g (~1534 rpm) for 7 minutes at 4ºC. Discard supernatant.
4. Resuspend pellet with Extraction Reagent plus inhibitors (see Reagent Preparation).
5. Vortex and incubate on ice for 30 minutes.
6. Centrifuge at 16,000 x g (~12,274 rpm) for 20 minutes at 4ºC.
7. Place the supernatants into a clean tube.
8. The supernatants may be aliquoted and stored at or below -20ºC or used immediately in the assay.

Serum and Plasma Preparation

1. Collect whole blood in either clotting tubes for serum or EDTA tubes for plasma.
2. Allow serum to clot for 30 minutes.
3. Centrifuge at 1000 x g for 15 minutes at 4ºC.
4. Place supernatants into a clean tube.
5. The supernatant may be aliquoted and stored at or below -20ºC, or used immediately in the assay.

ASSAY PROCEDURE

Refer to the Assay Layout Sheet to determine the number of wells to be used.
Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

1. Pipet 100 µL of the assay buffer into the S0 (0 pg/mL standard) wells.
2. Pipet 100 µL of Standards #1 through #6 to the bottoms of the appropriate wells.
3. Pipet 100 µL of the samples to the bottoms of the appropriate wells.
4. Seal the plate. Incubate for 1 hour shaking* at room temperature.
5. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 5 more times for a total of 6 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Pipet 100 µL of yellow antibody into each well except the blank.
7. Seal the plate. Incubate for 1 hour shaking* at room temperature.
8. Wash as above (Step 5).
9. Add 100 µL of blue conjugate to each well except the blank.
10. Seal the plate. Incubate for 30 minutes on a plate shaker (~500 rpm) at room temperature.
11. Wash as above (Step 5).
12. Pipet 100 µL of substrate solution into each well.
13. Incubate for 30 minutes shaking* at room temperature.
14. Pipet 100 µL of stop solution into each well.
15. After blanking the plate reader against the substrate blank, read optical density at 450 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

*Shaking is preferably carried out on a suitable plate or orbital shaker set at a speed to ensure adequate mixing of the contents of the wells. The optimal speed for each shaker will vary and may range from 120-700rpm.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of Hsp27 (phospho-Ser78) in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4-parameter logistic curve fitting program. If data reduction software is not readily available, the concentrations can be calculated as follows:

1. Calculate the average Net OD for each standard and sample by subtracting the average blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD - Average Blank OD

2. Using linear graph paper, plot the average Net OD for each standard versus Hsp27 (phospho-Ser78) concentration in each standard.
   Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

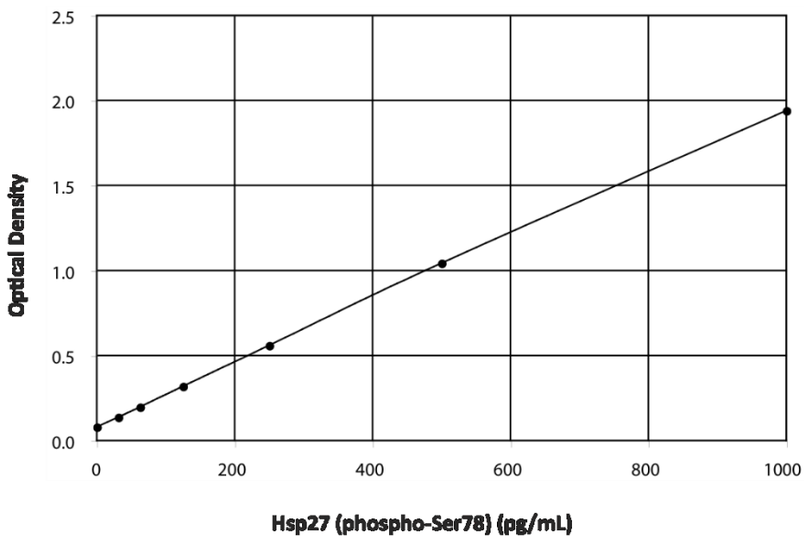
   Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Net OD	Hsp27 (phospho-Ser78) (pg/mL)
S0	0.080	0
S1	1.940	1000
S2	1.044	500
S3	0.560	250
S4	0.319	125
S5	0.197	62.5
S6	0.137	31.25
Unknown 1	0.845	396
Unknown 2	0.185	57

TYPICAL STANDARD CURVE



PERFORMANCE CHARACTERISTICS

Specificity

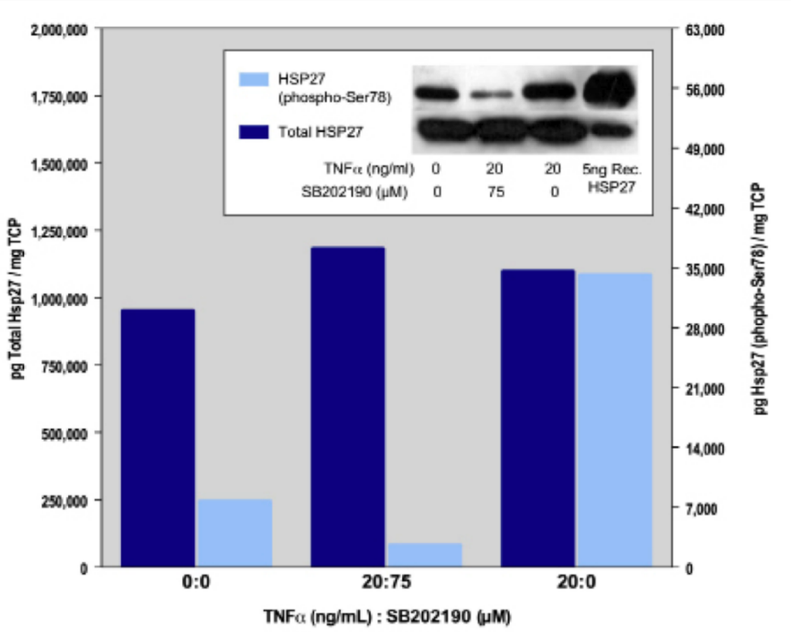
The cross reactivities for a number of related compounds were determined by diluting cross reactants in the assay buffer at several concentrations. These samples were then measured in the assay.

Compound	Cross Reactivity
Hsp27 (non-phosphorylated)	0.107%
Hsp25	<0.03%
Hsp40	<0.03%
Hsp70	< 0.03%
Hsp90	< 0.03%
αB-Crystallin	< 0.03%
αA-Crystallin	< 0.03%

Hsp27 (phospho-Ser78) has been shown to cross react at ≥ 100%. The percent cross reactivity calculation is relative to the phospho Hsp27 standard used in the assay. The percent cross reactivity varies depending on the percent phosphorylation and protein concentration of the cross reactant used and has been shown to vary between lots.

Stimulation Experiments

This experiment was adapted from a protocol outlined in reference 10. Human HeLa cells were treated with 75 µM SB202190, a cell permeable inhibitor of p38 MAPK, for 1 hour at 37ºC, followed by treatment with 20 ng/mL TNF-α, an inducer of Hsp27 phosphorylation, for 30 minutes at 37ºC. Cells were washed 3 times in HBSS and lysed in the 1X extraction reagent. Total cellular protein for each sample was determined using a BCA protein assay. Approximately 25 µg of total cellular protein was run on an 8-16% Tris-glycine gradient gel. Proteins were then transferred to a nitrocellulose membrane and probed with antibodies against total Hsp27 (Cat. # SPA-803) and Hsp27 (phospho-Ser78) (Cat. # SPA-523PU). The same lysates were also diluted in the assay buffer and run in this kit, as well as a kit specific for total Hsp27, independent of phosphorylation.



Sensitivity

Sensitivity was calculated as the ratio of the mean OD plus 2 standard deviations of twenty-four replicates of the 0 pg/mL standard to the mean of twenty-four replicates of the lowest standard, multiplied by the concentration of that standard (31.25 pg/mL). This value was determined to be 4.30 pg/mL.

Linearity

A buffer sample containing Hsp27 (phospho-Ser78) was serially diluted 1:2 in assay buffer and measured in the assay. The results are shown in the table below.

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	----------	783 pg/mL	---------
1:2	392 pg/mL	399 pg/mL	102 %
1:4	196 pg/mL	205 pg/mL	105 %
1:8	97.9 pg/mL	99.4 pg/mL	102 %
1:16	48.9 pg/mL	48.5 pg/mL	99 %

Precision

Intra-assay precision was determined by assaying 20 replicates of three buffer controls containing Hsp27 (phospho-Ser78) in a single assay.

pg/mL	%CV
406	1.9
109	2.3
52	4.1

Inter-assay precision was determined by measuring buffer controls of varying Hsp27 (phospho-Ser78) concentrations in multiple assays over several days.

pg/mL	%CV
396	3.1
105	3.9
54	6.1

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NOTES

NOTES

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