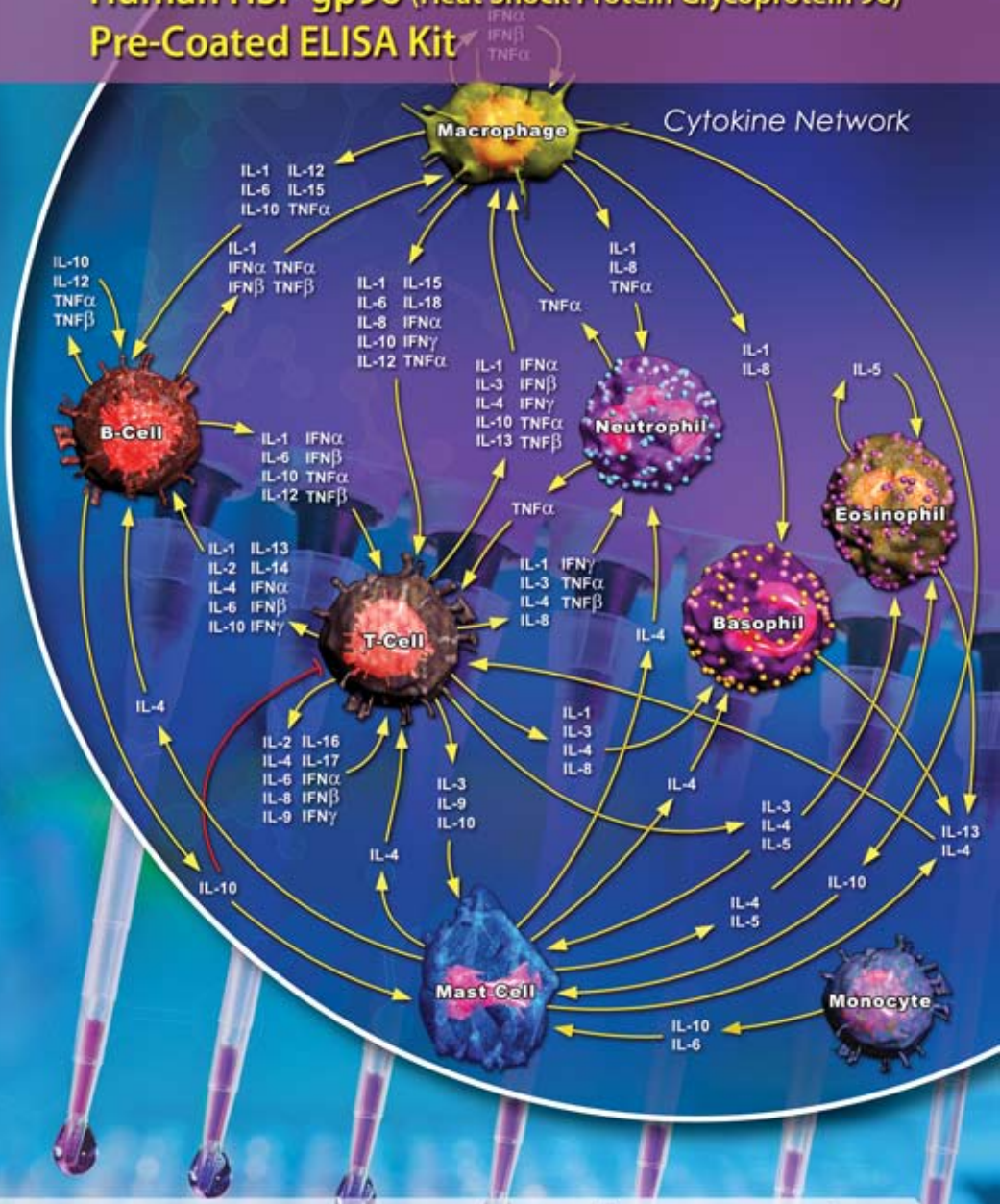
Human HSP gp96 (Heat Shock Protein Glycoprotein 96) Pre-Coated ELISA Kit



USER MANUAL

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Human HSP gp96

(Heat Shock Protein Glycoprotein 96)

Pre-Coated ELISA Kit

Catalog No: 90-2227
1 × 96 well Format (96 tests)
Detection Range: 0.313 – 20 ng/ml
Sensitivity: < 0.188 ng/ml

This immunoassay kit allows for the in vitro quantitative determination of Human HSP gp96 concentrations in serum, plasma and other biological fluids.

This kit is for Research Use Only. Not for use in diagnostic/therapeutics procedures.

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I. BACKGROUND

HSPs (Heat shock proteins) are a family of stress-inducible proteins, the expression and activity of which are highly regulated by the extracellular microenvironment. The HSP90 isoforms share common biological functions in cell survival and proliferation. It has been reported that high expression levels of HSPgp96, also known as gp96 and Grp94, are correlated with cancer cell survival and epithelial ovarian cancer. Suppression of HSP90B1 expression can greatly reduce cell survival and biological function. HSPgp96 displays unique immune-regulatory activity by loading antigens onto molecules of the MHC system for presentation to immune competent cells. HSPgp96 has a close relationship with antitumor immunity. In addition, it acts as a molecular chaperone and forms a complex with tumor antigens before the antigen-presenting cells incorporate the antigen.

II. OVERVIEW

This assay employs an antibody specific for Anti-human HSP gp96 coated on a 96-well plate. Standards and samples are pipetted into the wells and HSP gp96 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human HSP gp96 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of HSP gp96 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

III. ADVANTAGES

Multiple samples can be analyzed in a low volume, high-throughput format. Full analysis can be complete in 2 hours.

IV. STORAGE

Kit can be stored in 4°C, if you are using within a week.

If you are using within 6 months, lyophilized standard can be stored in -20°C and other components at 4°C.

Kit Components

Item	Specifications	Storage
96 well Strip ELISA Plate	8 × 12 well	4°C
Lyophilized Standard	2 vials	-20°C
Sample and Standard Dilution Buffer	20 ml	4°C
Biotinylated Detection Antibody for h HSPgp96	120 µl	4°C (Protected from light)
Antibody Dilution Buffer	10 ml	4°C
HRP Conjugated Streptavidin	120 µl	4°C (Protected from light)
Streptavidin Dilution Buffer	10 ml	4°C
TMB Substrate	10 ml	4°C
Stop Solution	10 ml	4°C
25X Wash Buffer	30 ml	4°C
Plate Sealer	5 pieces	
Product Manual	1	

Material Required, (Not Supplied)

Microplate Reader
37°C Incubator
Plate Reader
Multi Chanel Pipette and disposable tips
Eppendorf Tubes
Deionized Water

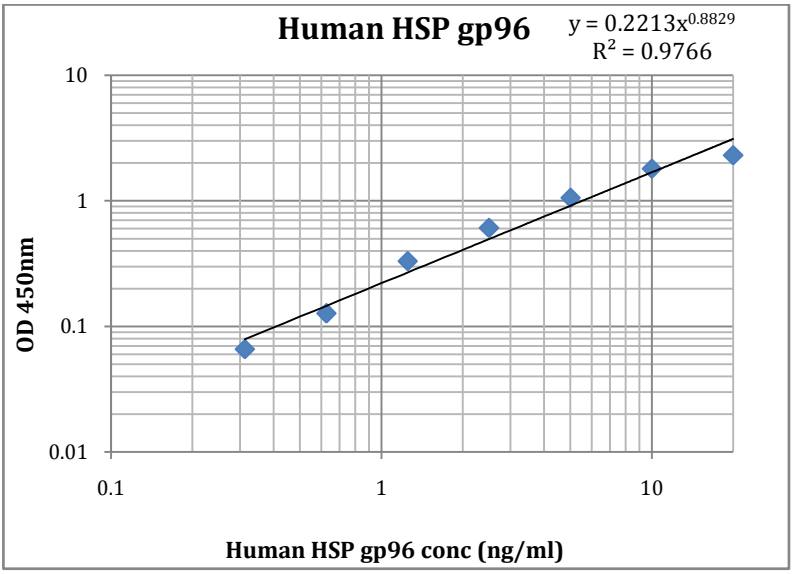
V. PRECAUTIONS FOR USE

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.
2. After opening and before using, keep plate dry.
3. Before using the Kit, spin tubes and bring down all components to the bottom of tubes.

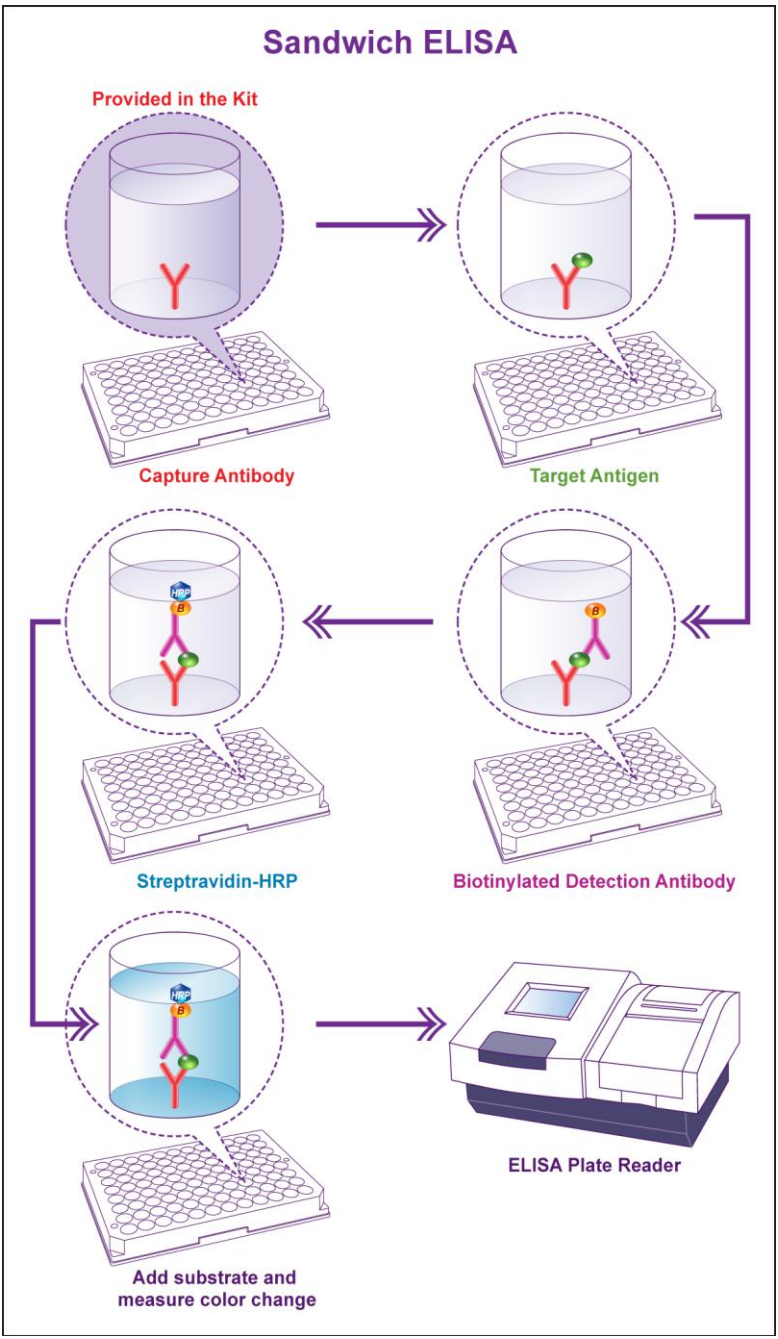
4. Storage TMB reagents avoid light.
5. Washing process is very important, not fully wash easily cause a false positive.
6. Duplicate well assay is recommended for both standard and sample testing.
7. Don't let Micro plate dry at the assay, for dry plate will inactivate active components on plate.
8. Don't reuse tips and tubes to avoid cross contamination.
9. Avoid using the reagents from different batches together.

VI. STANDARD CURVE

Human HSPgp96 Standard Curve is shown below.



X	ng/ml	20	10	5	2.5	1.25	0.625	0.312	0
Y	O.D.450	2.343	1.838	1.09	0.642	0.363	0.159	0.098	0.032


Sandwich ELISA
Provided in the Kit
Capture Antibody
Target Antigen
Biotinylated Detection Antibody
Streptravidin-HRP
ELISA Plate Reader
Add substrate and
measure color change

VII. REAGENT PREPARATION AND STORAGE

Included buffers and reagents are optimized for use with this kit. Substitution with other reagents is not recommended and may not give optimal results.

1. **Prepare Standard Curve:** One hour before the experiment.
   a. Quick spin down one vial of lyophilized standard. ***(DO NOT dilute standard directly on the plate)***. Add 1ml of sample/standard dilution buffer into one of the standard tube. Incubate at room temp. for 10 min. Mix thoroughly by vortex. Stock Standard concentration is 20 ng/ml.
   b. Label 6 eppendorf tubes with 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml respectively. Add 0.3 ml of sample/standard dilution buffer into each tube. Add 0.3 ml of stock standard (20 ng/ml) into 1st tube and mix thoroughly. Transfer 0.3 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.3 ml from 2nd tube to 3rd tube mix thoroughly, and so on.

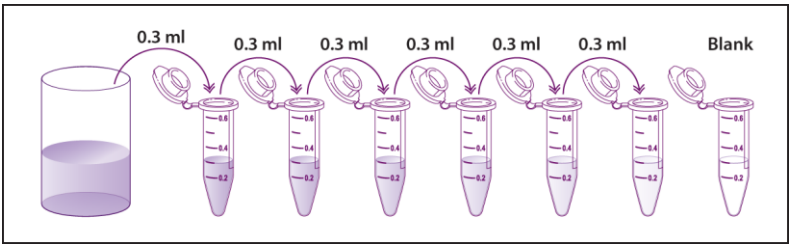


Fig-1: Dilution tubes

Note: *Standard Solutions are best used within 2 hrs. Standard solution should be stored at 4°C for up to 12 hrs. or store at -20°C for up to 48 hrs. Avoid repeated freeze-thaw.*

2. **Sample Preparation and storage:** Test samples should be collected, analyze immediately (within 2 hrs.) or aliquot and store at -20°C for long term. Avoid multiple freeze-thaw cycles.
   a. **Cell culture supernatants**: Centrifuge to remove precipitate, analyze immediately or aliquot and store at -20°C.

b. **Serum**: Coagulate the serum at room temp about 1 hr. Centrifuge approximately 1000 × g for 15 min. Analyze serum immediately or aliquot and store at -20°C.

c. **Plasma:** Collect plasma with heparin or EDTA as the anti-coagulant. Centrifuge for 15 min at 2-8°C at 1500 × g within 30 min of collection. For eliminating the platelet effect, suggesting that further centrifugation for 10 min at 2-8°C at 10,000 × g. Analyze immediately or aliquot and store frozen at -20°C.

d. **Tissue Homogenates**: For general information, hemolytic blood may affect the results, you should rinse the tissues with ice cold PBS (0.01M, pH 7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then minced to small pieces. This will be homogenized in PBS in a cold glass homogenizer. *(Volume depends on the weight of the tissue, 1gram of tissue requires 9 ml of ice cold PBS with protease inhibitor)*. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze- thaw cycle. Homogenates are then centrifuged for 5 min. at 5000 × g to get the supernatant.

Note: *Samples to be used within 5 days may be store at 4°C, otherwise sample should be stored at -20°C (< 1 month) or -80°C (<2 months) to avoid loss of bioactivity and contamination. Hemolyzed samples are not suitable for use in this Assay.*

e. End user should estimate the concentration of the target protein in the test samples first, then select proper dilution factor to make the diluted target protein concentration falls the optimal detection range of the kit. Dilute the samples with the provided dilution buffer. Several trials may be necessary in practice. The test sample should be well mixed with the dilution buffer. Standard curve and sample should be made before the experiment.

High target protein concentration 200-2000 ng/ml: Dilute 1:100 (add 1 µl of sample into 99 µl of sample/ standard dilution buffer).

Medium target protein concentration 20-200 ng/ml: Dilute 1:10 (add 10 µl of sample into 90 µl of sample/ standard dilution buffer).

Low target protein concentration 0.312-20 ng/ml: Dilute 1:2 (add 50 µl of sample into 50 µl of sample/ standard dilution buffer).

Very low target protein concentration < 0.312 ng/ml: Do not dilute, use 100 µl of sample or 1:2 dilution.

3. **Preparation of Biotin detection antibody working solution:** Prepare within one hour before the experiment. Calculate total volume working solution required. (0.1 ml/ well × number of wells. Add 100-200 µl extra).

   Dilute Biotin detection antibody with antibody dilution buffer at 1:100 and mix thoroughly. (*i.e.* add 1 µl of Biotin conjugated detection antibody into 99 µl of antibody dilution buffer).

4. **Preparation of HRP-Streptavidin Conjugate (SABC) working solution:** Prepare within 30 min before the experiment. Calculate total volume working solution required. (0.1 ml/well × number of wells. Add 100- 200 µl extra).

   Dilute SABC with SABC dilution buffer at 1:100 and mix thoroughly. (*i.e.* add 1 µl of SABC into 99 µl of SABC dilution buffer).

5. **Preparation of 1 X Wash buffer:** Prepare 1 X Wash buffer by diluting 25X Wash buffer in sterile water. Diluted Wash buffer may be stored at 4°C, however we recommend preparing fresh 1X wash buffer for each experiment.

   For example: *10 ml of 25X Wash buffer in 240 ml of sterile water.*

VIII. ASSAY PROCEDURE

Before starting the experiment, equilibrate the SABC working solution and TMB substrate for at least 30 min at room temp. When diluting samples and reagents, they should be mixed completely and evenly. It is recommended to plot a standard curve for each test.

** If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.*

1. Set standard, test sample and blank (control zero) wells on the pre-coated plate and then record their position. It is recommended to measure each standard and sample in duplicate. ***Note:*** *Wash plate twice before adding standard, sample and blank into the well.*
2. Add 0.1 ml of standard (20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml. 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml, control zero dilution buffer) into standard well.
3. Add 0.1 ml of diluted samples into test sample wells.
4. Seal plate with a cover and incubate at 37°C for 90 min.
5. Remove the cover and discard samples and standard solution by tapping plate on an absorbent paper. ***Note:*** *DO NOT let the wells completely dry any time. DO NOT wash plate.*
6. Add 0.1 ml of Biotin-detection antibody working solution into the above wells (Standards, control zero and samples).
7. Seal plate with cover and incubate at 37°C for 60 min.
8. Remove the cover, and wash plate 3 times with 1X wash buffer.
9. Add 0.1 ml of SABC working solution into each well. Cover the plate and incubate at 37°C for 30 min.
10. Remove the cover and wash plate 5 times with 1X wash buffer. Each time let the wash buffer stay in the well for 1-2 min.
11. Add 90 µl of TMB substrate into each well, cover the plate and incubate at 37°C in dark within 15-30 min. *(**Note**: This incubation time is for reference use only. The optimal time should be determined by end user)*. The shades of blue can be seen in the first 3-4 wells, only on most concentrated standards. Other wells show no obvious color.
12. Add 50 µl of stop solution into each well and mix thoroughly. Color will change into yellow immediately.
13. Read O.D. absorbance at 450 nm in a micro-plate reader immediately after adding the stop solution.

14. Calculation: Relative O.D. 450 = O.D. for each well – O.D. 450 control zero well. The Standard curve can be plotted as the relative O.D. 450 of each standard solution in Y axis vs. the respective concentration of the standard in X axis. Concentration of the samples can be incorporated from the standard curve. If the samples were diluted, multiply the dilution factor to the concentration.

Table-1

	Standard 1	Standard 2	3	4	5	6	7	8	9	10	11	12
A	**20 ng/ml**	**20 ng/ml**										
B	**10 ng/ml**	**10 ng/ml**										
C	**5 ng/ml**	**5 ng/ml**										
D	**2.5 ng/ml**	**2.5 ng/ml**										
E	**1.25ng/ml**	**1.25ng/ml**										
F	**0.625ng/ml**	**0.625ng/ml**										
G	**0.312ng/ml**	**0.312ng/ml**										
H	**0**	**0**										

IX. REFERENCES

1. The Role of Heat Shock Protein 90B1 in Patients with Polycystic Ovary Syndrome.
   PMID: 27046189

2. Proteomic Investigation on Grp94-IgG Complexes Circulating in Plasma of Type 1 Diabetic Subjects.
   PMID: 26167512

3. Expression of heat-shock protein gp96 in gallbladder cancer and its prognostic clinical significance.
   PMID: 25973087

X. TROUBLE SHOOTING

Problem	Probable Cause	Suggestion
No signal	Forgot to add all components.	Prepare check list and add the components in the correct order.
Low signal	Not enough lysates per well.	Check the protein concentration. Add more lysates.
High background	Washing is not sufficient.	Wash plates thoroughly after incubation with Streptavidin-HRP secondary

XI. SPECIFICITY

This assay has high sensitivity and excellent specificity for detection of HSPgp96. No significant cross-reactivity or interference between HSP gp96 and analogues was observed.

Note: Limited by current skills and knowledge, it is impossible for us to complete the cross- reactivity detection between HSP gp96 and all the analogues, therefore, cross reaction may still exist.

XII. RECOVERY

Matrices listed below were spiked with certain level of HSP gp96 and the recovery rates were calculated by comparing the measured value to the expected amount of AFP in samples.

Matrix	Recovery range (%)	Average (%)
Serum (n=5)	89-105	97
EDTA plasma (n=5)	86-100	93
heparin plasma (n=5)	86-104	95

XIII. LINEARITY

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of AFP and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	**1:2**	**1:4**	**1:8**	**1:16**
serum(n=5)	86-105%	88-104%	91-104%	91-104%
EDTA plasma(n=5)	88-96%	86-92%	82-96%	83-99%
heparin plasma(n=5)	81-95%	81-96%	80-100%	87-98%

XIV. PRECISION

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level HSP gp96 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level AFP were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/meanX100 Intra-Assay: CV<8% Inter-Assay: CV<10%

XV. STABILITY

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 10% within the expiration date under appropriate storage condition.

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.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