Anti-HSP60 IgG/A/M (human), ELISA kit

For the detection and quantitation of antibodies to Hsp60 in serum.

Catalog Number: ADI-EKS-650

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES

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A. INTRODUCTION

ASSAY DESIGN

The Anti-HSP60 IgG/A/M (human), ELISA kit provides a method to detect and quantitate antibodies to human Hsp60 in serum samples from human origin. This assay allows for reproducible, accurate and precise determination of IgG, IgA and IgM antibodies (total) to human Hsp60 in serum.

Enzo Life Sciences' Anti-HSP60 IgG/A/M (human), ELISA kit uses recombinant human Hsp60 bound to the wells of the Rec. Human Hsp60 Immunoassay Plate to bind anti-human Hsp60 antibodies present in human serum. The captured anti-human Hsp60 antibodies are detected with a hydrogen peroxidase conjugated goat polyclonal antibody specific for human IgG, IgA and IgM molecules. The assay is developed with tetramethylbenzidine substrate producing a blue color in proportion to the amount of captured anti-human Hsp60 antibodies. The color development is stopped with acid stop solution which converts the endpoint color to yellow. The intensity of the color is measured in a microplate reader at 450nm.

Traditional methods for detection and quantitation of anti-human Hsp60 antibodies were accomplished by using prescreened serum samples with a high level of anti-human Hsp60. These samples were assigned a concentration of 1000 arbitrary units/mL (Aunits/mL) and were used to generate standard dose-response curves from which antibody levels in test samples were determined.

Enzo Life Sciences' Anti-HSP60 IgG/A/M (human), ELISA kit uses a calibrated standard of anti-human Hsp60 (lgG/A/M) isolated from pooled human sera to generate a standard curve. The kit provides researchers with a rapid, reliable and standardized method to measure the levels of anti-human Hsp60 in human serum samples by interpolating absorbance readings from the standard curve.

SCIENTIFIC OVERVIEW

Heat shock protein 60 (Hsp60) is a molecular chaperone that participates in the folding of mitochondrial proteins and facilitates proteolytic degradation of misfolded or denatured proteins. Hsp60 is strongly dependent upon its co-chaperone, Hsp10, which binds the molecule and regulates its substrate binding and ATPase activity.[1,2,3] Hsp60 is rapidly upregulated by a range of cellular insults including oxidative stress, viral infection, chemical exposure, increased temperature, irradiation etc. Hsp60 is mainly located in the mitochondria but changes in the intracellular location and cell surface expression have been reported.[4,5,6,7,8]

The Hsp60 family of proteins, which includes GroEL from *E. coli* and Hsp65 from *mycobacteria*, are highly conserved through evolution and an autoimmune reaction may be evoked because of the immunological cross-reaction between self Hsp60 and microbial pathogens.[9,10] Human Hsp60 also shares sequence homology with a wide range of autoantigens and the extent of this homology may contribute to autoimmunity.[11] Autologous Hsp60, when expressed or released from stressed autologous cells, may serve as a danger signal for the innate immune system to mount an adaptive response. Both B and T cell responses to autologous Hsp60 have been reported to occur in a variety of inflammatory conditions. Anti-Hsp60 antibody levels have been studied in the peripheral circulation of normal controls and in the aging process.[12,13]

Hsp60 has become a focus of interest as a potential autoantigen during chronic inflammation, atherosclerosis and autoimmune diseases and anti-human Hsp60 levels have been measured in a variety of medical conditions. Significant increases in Hsp60 autoantibody levels have been shown to precede the appearance of glucose intolerance and diabetes in cystic fibrosis patients.[14] Elevated levels of circulating anti-human Hsp60 antibodies were found to be present in gastric atrophy patients and in patients with lymphoma of mucosa associated lymphoid tissue (MALT) type which is closely linked to host immune responses to *H. pylori*.[15,16] Higher levels of human and bacterial Hsp60 antibodies have been measured in glaucoma patients.[17,18] Increases of anti- Hsp60 antibodies have been reported in 43% of osteosarcoma patients 19. Levels of anti-Hsp60 antibodies are associated with the presence and severity of coronary artery disease and their simultaneous presence with high levels of antibodies to *Chlamydia pneumoniae* substantially increases the risk for disease development.[20,21,22,23,24,25,26,27] Recent data indicates that promoter polymorphism at position -174 of the IL-6 gene may be one of the genetic factors associated with high levels of autoantibodies to Hsp60.[28] Ongoing research is required to investigate the complex relationship between circulating human Hsp60 and anti-human Hsp60 in the potentiation or prevention of pathogenesis.

ASSAY PROCEDURE SUMMARY

1. Bring to room temperature: **Rec. Human Hsp60 Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent 1**, **TMB Substrate** and **Stop Solution 2**.
2. Centrifuge **Anti-Human Hsp60 Standard** before removing cap. **Caution!** This component is derived from human serum. **Treat as biohazard.**
3. Dilute **Anti-Human Hsp60 Standard** and samples in **Sample Diluent 1**.
4. Add 100µL prepared standards and samples in duplicate to wells of **Rec. Human Hsp60 Immunoassay Plate**. Cover immunoassay plate.
5. Incubate plate at room temperature for 2 hours.
6. Wash wells 4X with 1X Wash Buffer.
7. Add 100µL **Anti-Human GAM:HRP Conjugate** to each well. Cover immunoassay plate.
8. Incubate plate at room temperature for1 hour.
9. Wash wells 4X with 1X Wash Buffer.
10. Add 100µL **TMB Substrate** to each well.
11. Incubate at room temperature for 15 minutes.
12. Add 100µL **Stop Solution 2** to each well.
13. Measure absorbance at 450nm.
14. Plot the anti-human Hsp60 (IgG/A/M) standard curve and calculate the anti-human Hsp60 sample concentrations.

B. MATERIALS

PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- **Caution!** The **Anti-Human Hsp60 Standard** *(part# 80-0950)* is derived from human serum. **Treat as biohazard.** Care should be taken in handling this material to minimize possible contamination with infectious agents present in human serum. The serum from which this product is derived was tested by an FDA approved method and found negative for HIV-1, HIV-2, HIV p24 Ag and non reactive to HbsAg, HVC-3 and STS. No known test method can offer complete assurance that Hepatitis B virus, Hepatitis C virus, HIV-1, HIV-2 or other infectious agents are absent.
- The **Stop Solution 2** *(part# 80-0377)* is a 1 Normal (1N) solution of acid. This solution is corrosive; please use caution when handling.
- The activity of the **Anti-Human GAM-HRP Conjugate** *(part# 80-1541)* is affected by nucleophiles such as azide, cyanide and hydroxylamine.

MATERIALS PROVIDED

The Anti-HSP60 IgG/A/M (human), ELISA kit contains the following components in sufficient quantities for 96 wells. These reagents are sufficient to assay one standard curve and 41 samples in duplicate or two standard curves and 34 samples in duplicate.

PART #	COMPONENT	SIZE	DESCRIPTION
80-0947	Rec. Human Hsp60 Immunoassay Plate	96 well plate	12 x 8 removable strips and frame. Pre-coated plate with recombinant human Hsp60 protein
80-0950	Anti-Human Hsp60 Standard	120µL	Human serum containing anti-human Hsp60 IgG, IgA, IgM antibodies
80-1624	Sample Diluent 1	100mL	Buffer to dilute standards and samples
80-1287	20X Wash Buffer	100mL	Concentrated solution of buffer and surfactant
80-1541	Anti-Human GAM-HRP Conjugate	10mL	Horseradish peroxidase conjugated polyclonal antibody specific for human IgA, IgG, IgM antibodies
80-0350	TMB Substrate	10mL	Stabilized tetramethylbenzidine substrate
80-0377	Stop Solution 2	10mL	Acid stop solution to stop color reaction

STORAGE OF MATERIALS

All reagents are stable as supplied at 4°C, until the kits expiry date.
Unused wells of the **Rec. Human Hsp60 Immunoassay Plate** should be resealed in the foil pouch provided and stored at 4°C until the kits expiry date.

MATERIALS REQUIRED BUT NOT PROVIDED

- Deionized or distilled water
- Precision pipettors capable of accurately delivering 1 to 1000µL
- Disposable pipette tips
- 5, 10, 25mL pipettes for reagent preparation
- 1L Graduated cylinder
- Squirt bottle, manifold dispenser, or automated microtiter plate washer
- Disposable polypropylene tubes
- Microtiter plate reader capable of measuring absorbance at 450nm
- Adhesive plate sealers or plastic wrap

C. PERFORMING THE ASSAY

CRITICAL ASSAY PARAMETERS AND NOTES

- The Assay Designs Anti-Human Hsp60 (total) ELISA Kit contains a pre-coated microtiter plate (**Rec. Human Hsp60 Immunoassay Plate**) with removable wells to allow assaying on two separate occasions.
- Run both standards and samples in duplicate.
- Include a standard curve each time the assay is performed.
- The following kit components should be allowed to warm to room temperature for at least 30 minutes prior to use: **Rec. Human Hsp60 Immunoassay Plate, 20X Wash Buffer, Sample Diluent 1, TMB Substrate, Stop Solution 2**.
- Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents are ready to use and all required strip-wells secured in the microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.
- Mix all reagents and samples gently, yet thoroughly, prior to use. Avoid foaming of reagents.
- To avoid cross contamination, change disposable pipette tips between the addition of each standard, samples, and reagents. Use separate reagent troughs/reservoirs for each reagent.
- This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated.
- Consistent, thorough washing of each well is critical. If using an automatic washer, check washing head before use. If washing manually, ensure all wells are completely filled at each wash. Air bubbles should be avoided.
- Exercise appropriate laboratory safety precautions when performing this assay.

NOTE: *The components in each kit lot number have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot numbers.*

SAMPLE PREPARATION

1. COLLECTION OF SERUM

   a) Collect whole blood using a serum separator tube.

   b) Allow samples to clot at room temperature for 30 minutes.

   c) Centrifuge at approximately 1000 x g for 10 minutes, taking precautions to avoid hemolysis.

   d) Remove serum. Transfer the serum to a labeled polypropylene tube. The serum collected is now ready for analysis using the Anti-Human Hsp60 (total) ELISA Kit.

   e) Alternatively, the serum sample can be frozen at ≤-20°C and assayed at a later date. It is recommended that the serum be aliquotted to convenient volumes prior to storing at ≤-20°C to avoid multiple freeze thaw cycles.

2. DILUTION OF SAMPLES

   Serum samples should be prepared as described in #1 above. Serum can be diluted 1:1000 (v/v) in **Sample Diluent 1** by a 2 step serial dilution (1:10 dilution followed by a 1:100 dilution). This is a suggested starting dilution only. Additional dilutions may be necessary to ensure that sample values are within the range of the standard curve. Users must determine the optimal sample dilutions for their particular experiments.

   a) Dilute prepared samples in **Sample Diluent 1.** Prepare at least 250 µL of diluted sample to permit assaying in duplicate.

   b) Mix thoroughly.

   c) Samples are now ready to be used in the Assay Procedure (see page 12). Samples may be left at room temperature while reagents are being prepared (see page 11).

REAGENT PREPARATION

NOTE: *All reagents should be freshly prepared prior to use. Once prepared, reagents should be kept at room temperature for the duration of the assay.*

NOTE*: The preparation of the reagents is based on using the complete 1 X 96 well assay, unless otherwise noted. If only a portion of the immunoassay plate is to be used, please store all components as previously described (see page 7).*

1. TEMPERATURE OF REAGENTS

   Bring the following reagents to room temperature prior to use:

   - **Rec. Human Hsp60 Immunoassay Plate** *(Part#: 80-0947)*
   - **Sample Diluent 1** *(Part#: 80-0948)*
   - **Wash Buffer** *(Part#: 80-1287)*
   - **TMB Substrate** *(Part#: 80-0350)*
   - **Acid Stop Solution** *(Part#: 80-0377)*

2. ANTI-HUMAN HSP60 STANDARD *(part#: 80-0950)*

   Caution: This standard is derived from human serum. Treat as biohazard.

   The **Anti-Human Hsp60 Standard** is used to generate a standard curve with 6 points, ranging from 7.81 - 250ng/mL.

   a) Centrifuge the **Anti-Human Hsp60 Standard** vial before removing cap.

   b) Label six (6) disposable 12 x 75mm tubes with the following standard values: 250ng/mL, 125ng/mL, 62.5ng/mL, 31.25ng/mL, 15.63ng/mL, 7.81ng/mL.

   c) Add 0.9mL of **Sample Diluent 1** to Tube #1 (250ng/mL).

   d) Add 0.5mL of **Sample Diluent 1** to Tube #2, 3, 4, 5 and 6.

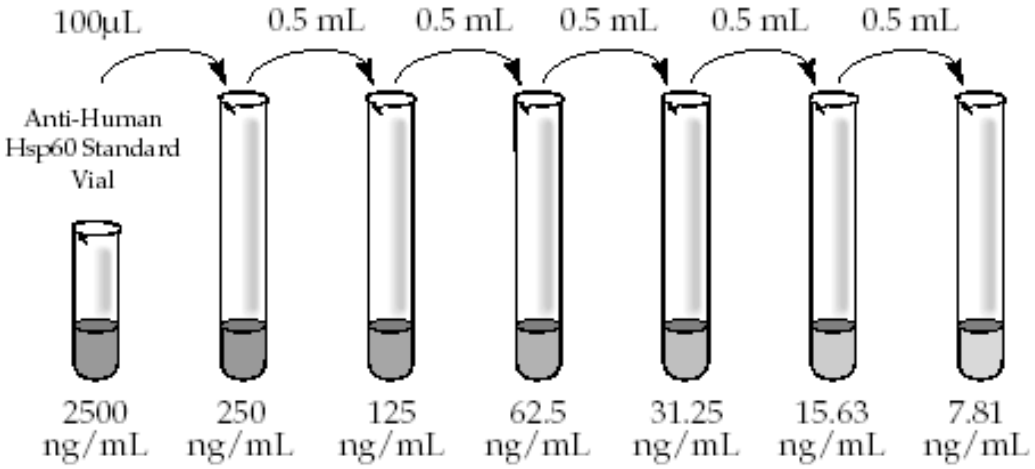
   e) Add 100µL of the **Anti-Human Hsp60 Standard** stock (2500ng/mL) to Tube #1 (250ng/mL).

   f) Mix gently.

g) Transfer 0.5mL from Tube #1 (250ng/mL) to Tube #2 (125ng/mL).

h) Mix gently.

i) Similarly, complete the dilution series to generate the remaining standards (0.5mL from Tube #2 to Tube #3, mix gently, etc) up to and including Tube #6.



j) Finally, add 0.5mL **Sample Diluent 1** to another 1.5mL disposable 12 x 75mm tube (Tube #7), which is the zero standard (0ng/mL).

NOTE: *Diluted standards should be used within 60 minutes of preparation.*

3. WASH BUFFER *(Part#: 80-1287)*

a) Bring the **20X Wash Buffer** to room temperature and swirl gently to dissolve any crystals that may have formed from storage.

b) Dilute the 100mL of **20X Wash Buffer** with 1900mL of deionized or distilled water. Once diluted, the 1X Wash Buffer is stable at room temperature for up to 4 weeks. For longer-term storage, the Wash Buffer should be stored at 4˚C.

NOTE*: 100mL of **20X Wash Buffer** has been provided in this kit, which is sufficient for the preparation of 2L of 1X Wash Buffer. The minimum required volume of 1X Wash Buffer is 310mL (if the complete plate is used at once). However additional 1X Wash Buffer is supplied to allow for multiple assays or alternative washing techniques.*

ASSAY PROCEDURE

1. DETERMINE THE REQUIRED NUMBER OF WELLS

   a) If less than 96 pre-coated microtitre wells are needed, remove the excess wells from the frame and return them to the foil pouch.

   b) Reseal the pouch containing the unused wells and store at 4°C.

2. ADDITION OF STANDARDS and SAMPLES

   a) Add 100 µL **(in duplicate)** of each of the following to appropriate wells:

   - Prepared anti-human Hsp60 standards (Tube#1 through Tube #6)
   - Samples (previously prepared - see Sample Preparation, page 9)
   - 0 Standard (Sample Diluent 1, which represents 0ng/mL)

   b) Cover wells with an adhesive plate sealer or plastic wrap and incubate at room temperature for 2 hours, preferably with gentle rocking or shaking.

3. WASHING

   a) Aspirate liquid from all wells.

   b) Add 400 µL of 1X Wash Buffer to each well, using a multi-channel pipette, manifold dispenser, automated microplate washer, or a squirt bottle.

   c) Repeat the aspirating and washing 3 more times, for a total of 4 washes.

   d) After the 4th addition of 1X Wash Buffer, aspirate the liquid from all wells. Invert the plate and carefully pat dry on clean paper towels.

4. ADDITION OF ANTI-HUMAN GAM-HRP CONJUGATE

a) Add 100 µL of the **Anti-Human GAM-HRP Conjugate** to each well.

b) Cover wells with a fresh adhesive plate sealer or plastic wrap and incubate at room temperature for 1 hour, preferably with gentle rocking or shaking.

c) Wash plate as described in Step #3.

5. ADDITION OF TMB SUBSTRATE AND STOP SOLUTION

a) Add 100 µL of the **TMB Substrate** to each well.

b) Incubate the plate at room temperature for approximately 15 minutes, preferably with gentle rocking or shaking.

c) Add 100 µL of the **Stop Solution 2** to each well in the same order that the **TMB Substrate** was added.

6. MEASURING ABSORBANCE

a) Set up the microplate reader according to the manufacturer's instructions.

b) Set wavelength at 450nm. If the reader is capable of measuring at dual wavelengths, set the correction wavelength at 540 or 570nm.

c) Measure the absorbance. If the plate cannot be read immediately, it should be covered and kept at room temperature. The absorbance should be read within 30 minutes of adding the stop solution

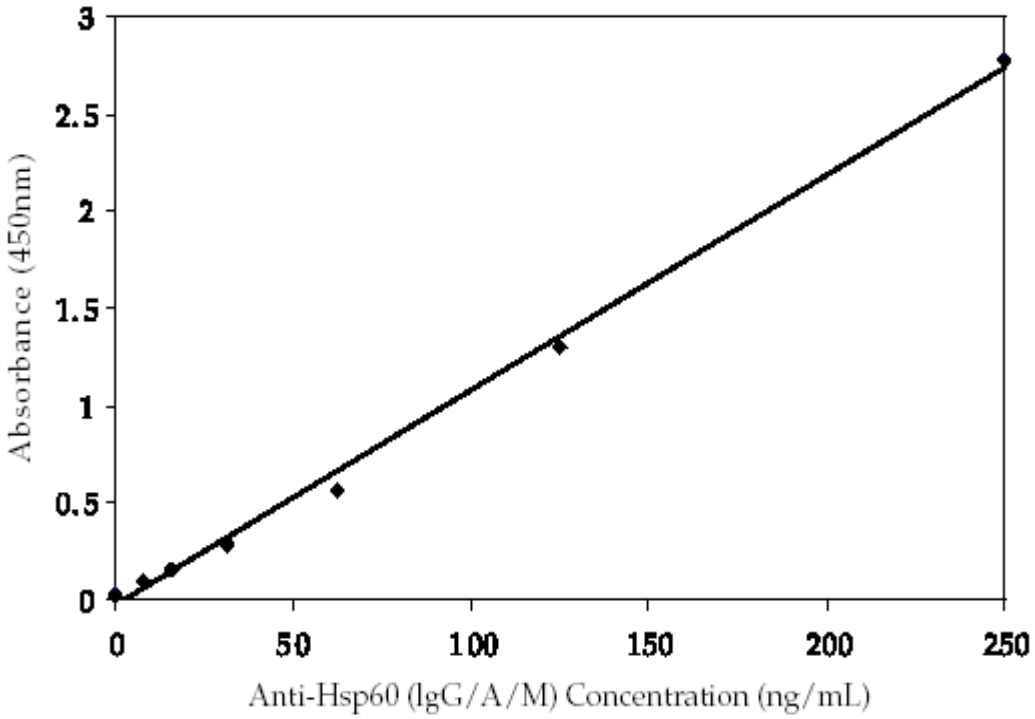
CALCULATION OF RESULTS – DETERMINATION OF ANTI-HUMAN HSP60 CONCENTRATIONS

1. Calculate the average of the duplicate absorbance measurements for each standard and sample.
2. Calculate the average of the duplicate absorbance measurements for the 0ng/mL anti-human Hsp60 standard (assay blank).
3. Subtract the average value obtained in Step#2 (0ng/mL anti-human Hsp60 standard (assay blank)) from the values obtained in Step#1 (standards and samples).
4. To generate the standard curve, plot the anti-human Hsp60 standard concentrations (ng/mL) on the X-axis and the corresponding absorbance measurements on the Y-axis. Determine the best fit line.
5. Interpolate the sample concentrations from the standard curve and multiply by the dilution factor for the final sample anti-human Hsp60 concentration. For example, if the sample was diluted 1:25 prior to assaying, the value generated from the standard curve must be multiplied by 25 to calculate the final sample anti-human Hsp60 (IgG/A/M) concentration.

NOTE: *Manufacturers of microplate readers usually offer accompanying software programs that will analyze data, plot standard curves and calculate sample concentrations. To set up the program for calculating the results, consult with the software instruction manual or contact the manufacturer of the microplate reader.*

D. ASSAY PERFORMANCE CHARACTERISTICS

TYPICAL ANTI-HUMAN HSP60 STANDARD CURVE



PERFORMANCE CHARACTERISTICS

1. SENSITIVITY

   To determine sensitivity of the assay, the mean absorbance value for sixteen replicates of Sample Diluent 1 (0ng/mL) was compared to the mean absorbance value for sixteen replicates of standard Tube #6 (7.81ng/mL). The detection limit was determined as the concentration of anti-human Hsp60 (IgG/A/M) measured at two standard deviations from the 0ng/mL standard along the standard curve. The sensitivity of the Anti-HSP60 IgG/A/M (human), ELISA was determined to be 2.88ng/mL.

2. PRECISION

a) Intra-Assay Precision (Within Run Precision)

To determine Intra-Assay Precision, samples containing low, medium and high concentrations of anti-human Hsp60 were assayed sixteen times on one plate. The Intra-Assay coefficient of variation of the Anti-Human Hsp60 (total) ELISA was determined to be <10%.

b) Inter-Assay Precision (Between Run Precision)

To determine Inter-Assay Precision, three samples containing low, medium and high concentrations of anti-human Hsp60 were assayed in eight individual assays. The Inter-Assay coefficient of variation of the Anti-Human Hsp60 (total) ELISA was determined to be <10%.

3. LINEARITY

To determine linearity, a sample containing 189.3ng/mL of anti-human Hsp60 was diluted five times 1:2 in Sample Diluent 1 and measured in the assay. The data was plotted graphically as actual anti-human Hsp60 concentration versus measured anti-human Hsp60 concentration. The line obtained had a slope of 0.948 and a correlation coefficient of 0.998.

LIMITATIONS OF THE ASSAY

- This assay has been validated for use with serum. Other sample types or matrices (e.g. tissue extracts, cell lysates, urine, cerebrospinal fluid, cell culture supernatant, etc.) may contain interfering factors that can compromise the performance of the assay, or produce inaccurate results.
- If serum samples generate greater values than the highest standard, the samples should be re-assayed at a higher sample dilution. Similarly, if samples generate lower values than the lowest standard, the samples should be re-assayed at a lower sample dilution.
- The use of assay reagents not provided in this kit or amendments to the protocol can compromise the performance of this assay.
- The components in each kit lot number have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot numbers.

E. REFERENCES

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NOTES

F. APPENDIX

APPENDIX – Rec. Human Hsp60 Immunoassay Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	62.5ng/mL										
B	Blank	62.5ng/mL										
C	0ng/mL	31.25ng/mL										
D	0ng/mL	31.25ng/mL										
E	250ng/mL	15.63ng/mL										
F	250ng/mL	15.63ng/mL										
G	125ng/mL	7.81ng/mL										
H	125ng/mL	7.81ng/mL										

REFERENCE

1. Bring to room temperature: **Rec. Human Hsp60 Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent 1**, **TMB Substrate** and **Stop Solution 2**.
2. Centrifuge **Anti-Human Hsp60 Standard** before removing cap. **Caution!** This component is derived from human serum. **Treat as biohazard.**
3. Dilute **Anti-Human Hsp60 Standard** and samples in **Sample Diluent 1**.
4. Add 100µL prepared standards and samples in duplicate to wells of **Rec. Human Hsp60 Immunoassay Plate**. Cover immunoassay plate.
5. Incubate plate at room temperature for 2 hours.
6. Wash wells 4X with 1X Wash Buffer.
7. Add 100µL **Anti-Human GAM:HRP Conjugate** to each well. Cover immunoassay plate.
8. Incubate plate at room temperature for1 hour.
9. Wash wells 4X with 1X Wash Buffer.
10. Add 100µL **TMB Substrate** to each well.
11. Incubate at room temperature for 15 minutes.
12. Add 100µL **Stop Solution 2** to each well.
13. Measure absorbance at 450nm.
14. Plot the anti-human Hsp60 (IgG/A/M) standard curve and calculate the anti-human Hsp60 sample concentrations.

Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

Warranty

Enzo Life Sciences International, Inc. makes no warranty of any kind, expressed or implied, which extends beyond the description of the product in this brochure, except that the material will meet our specifications at the time of delivery. Enzo Life Sciences International, Inc. makes no guarantee of results and assumes no liability for injuries, damages or penalties resulting from product use, since the conditions of handling and use are beyond our control.

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