# Human Heat Shock Protein 60 (HSP-60) ELISA
## Cat No: K12-1775

**Principle:**
The Human Heat Shock Protein 60 ELISA is sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human Heat Shock Protein 60 in samples. Standards or Samples are added to the microtiter well which is pre-coated with Human Heat Shock Protein 60 monoclonal Antibody. Biotinylated Human Heat Shock Protein 60 antibody is added to the microplate to form a complex. Subsequently Streptavidin-HRP conjugate is pipetted. After incubation and a washing step TMB Substrate A and B, are added. Blue color develops on incubation and the reaction is stopped with a Stop Solution to form a yellow color. The concentration of the Human Heat Shock Protein 60 in the samples is directly proportional to the yellow color developed in the wells.

**Intended Use:**
This Kit is used to assay the level of Human Heat Shock Protein 60 in Human serum and plasma samples. The Kit is For Laboratory / Research Use Only.

**Materials provided in the Kit:**
1. Anti-Human Heat Shock Protein 60 Coated Microtitre Plate (96 wells) – 1 no
2. Biotinylated Human Heat Shock Protein 60 Antibody – 1 ml
3. Human Heat Shock Protein 60 Standard (concentrated, 40 ng/ml ) – 0.5 ml
4. Streptavidin:HRP Conjugate – 6 ml
5. (30X) Wash Buffer – 20 ml
6. Standard Diluent – 3 ml
7. TMB Substrate A – 6 ml
8. TMB Substrate B – 6 ml
9. Stop Solution – 6 ml
10. Instruction Manual

**Materials to be provided by the End-User:**
1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Tubes to prepare standard/sample dilutions.
7. Timer.
8. Absorbent paper.
9. Incubator

**Storage Information:**
1. All reagents should be stored at 2ºC to 8ºC.
2. All the reagents and wash solutions are stable until the expiration date of the kit.
3. 30 minutes prior before use, bring all components to room temperature (18-25ºC). Store all the components of the kit at its appropriate storage condition after use.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

**Health Hazard Warnings:**
1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details.
2. To reduce the likelihood of blood-borne transmission of infectious agents, handle all samples in accordance with NCCLS regulations.

**Specimen Collection and Handling:**
Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.
1. The kit cannot test samples which contain $NaN_3$, because $NaN_3$ inhibits HRP activity.

2. Extract as soon as possible after specimen collection as per relevant procedure. The samples should be tested as soon as possible after the extraction. Alternately the extracted samples can be kept in -20°C. Avoid repeated freeze-thaw cycles.
3. **Serum-** Coagulate at room temperature for 10-20 minutes; centrifuge for 20-min at 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.
4. **Plasma-** Use EDTA or citrate plasma as an anticoagulant, mix for 10-20 minutes; centrifuge for 20-min at the 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.

***Note:*** *Grossly hemolyzed samples are not suitable for use in this assay.*

**Reagent Preparation (all reagents should be diluted immediately prior to use):**

1. Bring all reagents to Room Temperature prior to use.
2. To make 1X Wash Solution, add 10 ml of 30X Wash Buffer in 290 ml of DI water.

**Procedural Notes:**

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Human Heat Shock Protein 60. High Dose Hook Effect is due to excess of antibody for very high concentrations of Human Heat Shock Protein 60 present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Human Heat Shock Protein 60 concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
3. Avoid assay of Samples containing Sodium Azide ($NaN_3$), as it could destroy the HRP activity resulting in under-estimation of the amount of Human Heat Shock Protein 60.
4. It is recommended that all Controls and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to poor sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Controls and Samples.

**Assay Procedure:**

1) Bring all reagents to room temperature prior to use. It is strongly recommended that all Standards and Samples should be run in duplicates or triplicates. A standard curve is required for each assay.
2) Standards Dilution: Prepare the standards as per the table given below using the provided standard Concentration and Standard Diluent.

| Standard Concentration | Standard No | Dilution Particulars |
|---|---|---|
| 40 ng/ml | Standard, concentrated | Original Standard provided in the Kit |
| 20 ng/ml | Standard No.5 | 120 ul Original Standard + 120 ul Standard Diluent |
| 10 ng/ml | Standard No.4 | 120 ul Standard No.5 + 120 ul Standard Diluent |
| 5 ng/ml | Standard No.3 | 120 ul Standard No.4 + 120 ul Standard Diluent |
| 2.5 ng/ml | Standard No.2 | 120 ul Standard No.3 + 120 ul Standard Diluent |
| 1.25 ng/ml | Standard No.1 | 120 ul Standard No.2 + 120 ul Standard Diluent |

** refer accompanying sheet with the Standard, concentrated in the kit*

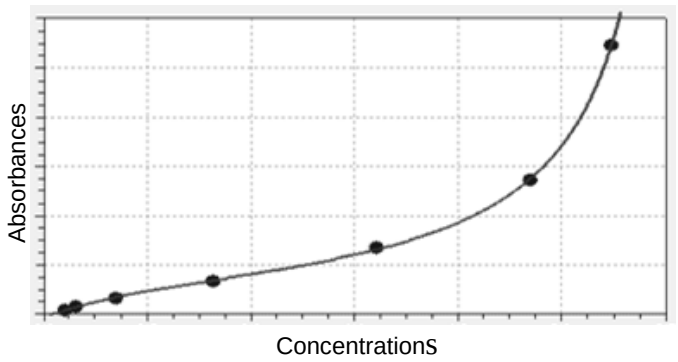
3) The quantity of the plates depends on the quantities of samples and standards to be tested. It is suggested to remove the number of strips required for the assay.
4) Pipette **50 ul** of **Standards** and **40 ul Samples** into the respective wells as mentioned in the work list.
*Note do not add the sample, Biotin Conjugate and Streptavidin-HRP to the blank well.*
*5)* Pipette **10 ul** of **Biotinylated Human Heat Shock Protein 60 Antibody** into each sample well.
*Do not pipette into the blank and standards wells.*

*6)* Pipette **50 ul** of **HRP Conjugate** into each sample and standards well.
*Do not pipette into the Blank well.*
7) Cover the plate and incubate for 1 hour at 37°C in the incubator.
8) Aspirate and wash plate 4 times with **1X Wash Buffer** and blot residual buffer by firmly tapping the plate on an absorbent paper. Wipe off any liquid from the bottom of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.
9) Add **TMB Substrate A 50 ul** and **TMB Substrate B 50 ul** respectively to each well. Gently mix.
10) Incubate for 10 min at 37°C in dark.
11) Pipette **50 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.
12) Read the absorbance at 450 nm within 15 minutes after adding the Stop Solution. Blank the zero standard for net absorbance.

**Calculation of Results:**
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Use the Net Absorbance (Absorbance of Standard/Sample - Absorbance of Blank) to calculate the Mean Absorbances. Using standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a cubic spline curve-fit or a polynomial regression to the 2$^{nd}$ order is best recommended for automated results.

**Typical Graph**




**Precautions:**
Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

**Performance Characteristics:**
Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

**Sensitivity:**
Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was found to **1.0 ng/ml.**

**Specificity:**
The antibodies used in the kit for capture and detection are specific for Human Heat Shock Protein 60.

**Assay Range:**
**1.25 ng/ml to 20 ng/ml**

**Precision:**
Intra-Assay: CV<10%
Inter-Assay: CV<12%

**Linearity:**
The Linearity of the kit was assayed by testing samples spiked with appropriate concentration of Human Heat Shock Protein 60 and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

| Sample | 1:2 | 1:4 | 1:8 |
|---|---|---|---|
| serum (n=5) | 85-105% | 86-109% | 83-112% |
| EDTA plasma (n=5) | 84-106% | 85-117% | 83-118% |
| heparin plasma (n=5) | 83-99% | 80-95% | 82-93% |

**LIMITED WARRANTY**

KinesisDx does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the product; against defects in products or components not manufactured by KinesisDx, or against damages resulting from such non-KinesisDx made products or components. KinesisDx passes on to customer the warranty it received (if any) from the maker thereof of such non-KinesisDx made products or components. This warranty also does not apply to product to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by KinsisDx.

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# Human Heat Shock Protein 60 (HSP-60) ELISA

## ASSAY PROCEDURE

| | | | |
|---|---|---|---|
| 1 | Bring all reagents to room temperature before use. | | |
| 2 | Pipette Standards 1 - 6<br>Samples | 50 ul | 40 ul |
| 3 | Pipette Human Heat Shock Protein 60 Biotin Detection Antibody | | 10 ul |
| 4 | Pipette Streptavidin :HRP Conjugate | 50 ul | 50 ul |
| 5 | Incubate | 60 minutes (37ºC) | |
| 6 | 1X Wash Buffer | Decant, 4 x 300 ul | |
| 7 | Pipette TMB Substrate (A) | 50 ul | 50 ul |
| 8 | Pipette TMB Substrate (B) | 50 ul | 50 ul |
| 9 | Incubate in the dark | 10 minutes (37ºC) | |
| 10 | Pipette Stop Solution | 50 ul | 50 ul |
| 11 | Measure 450 within 15 mins | | |

## Troubleshooting:

| Problem | Possible cause | Investigation/Actions |
|---|---|---|
| High Absorbances | 1. Cross-contamination from other specimens | > Repeat assay taking care when washing and pipetting. |
| | 2. Insufficient or inefficient washing or reading | > Check washer efficiency |
| | 3. Wavelength of filter not correct. | > Check that the wavelength is 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm. |
| | 4. High assay background. | > Repeat assay and include a well that contains only sample diluent or sample absorbent (i.e. a blank well). |
| | 5. Contaminated TMB | > Check that TMB is colorless or faint blue. |
| | 6. Incubation time too long or incubation temperature too high. | > Check incubation time and temperature.<br>> Check incubator is at the correct temperature. |
| | 7. Incorrect dilution of serum | > Repeat assay, ensuring correct serum dilution is used. |
| Low Absorbances | 1. Incubation time too shot or incubation temperature too low. | > Ensure time and temperature of assay incubation are correct.<br>> Check incubator is set at the correct temperature. |
| | 2. Incorrect dilution or pipetting of sera | > Repeat assay ensuring correct dilutions and volumes are used.<br>> Ensure controls are sufficiently mixed. |
| | 3. Incorrect filter wavelength. | > Check the wavelength is set at 450nm. If a dual wavelength spectrophotometer is available, set the reference filter between 600-650nm. |
| | 4. Contaminated Conjugate solution. | > Dispense conjugate directly from the bottle using clean pipette tip; avoid transferring Conjugate to another container if possible.<br>> Do not return unused Conjugate to bottle.<br>> Ensure all pipettes and probes used to dispense the Conjugates are clean and free from serum, detergent and bleach. |
| | 5. Kit has expired. | > Check expiration date of kit and do not use if expired. |
| | 6. Air blank reading high. | Investigate causes of high background absorbance. |
| | 7. Incorrect storage of kit. | > Ensure kit is stored at 2-8ºC,plate is sealed in foil pouch and desiccant sachet is blue/purple. |
| | 8. Kit reagents not equilibrated at room temperature | > Allow sufficient time for reagents to equilibrate to room temperature prior to assay. |
| | 9. Incorrect reagents used. | > Check the reagents used match those listed on the specification sheet. |
| | 10. Over washing of plate (e.g. inclusion of a long soak step). | > Repeat assay using recommended wash procedure. |
| Poor Duplicates | 1. Poor mixing of samples. | > Mix reagents gently and equilibrate to room temperature. |
| | 2. Poor pipette precision | > Calibration may need to be checked.<br>> Check pupating technique-change pipette tip for each sample and ensure excess liquid is wiped from the outside of the tip. |
| | 3. Addition of reagents at inconstant timing intervals; reagent addition takes too long, air bubbles when adding reagents. | > Use consistent timing when adding reagents.<br>> Ensure all dilutions are made before commencing addition to plate.<br>> Improve pipetting technique and skill. |
| | 4. Inefficient washing - Wash buffer left in wells, inconsistent washing, inadequate washing. | > Tap out wash buffer after washing.<br>> Check wells are sufficiently and uniformly filled and aspirated when washing. |
| | 5. Reader not calibrated or warmed up prior to plate reading. | > Check reader precision<br>> Check reader manual to ascertain warm up time of instrument. |
| | 6. Optical pathway not clean | > Gently wipe bottom of plate.<br>> Check reader light source and detector are clean. |
| | 7. Spillage of liquid from wells | > Repeat assay, taking care not to knock the plate or splash liquid |
| | 8. Serum samples exhibit microbial growth, haemolysis or lipaemia. | > It is not recommended to use serum samples exhibiting microbial growth, haemolysis or lipaemia. |
| | 9. Uneven well volumes due to evaporation. | > Cover plate with a lid or plate sealer (not provided). |
| All wells yellow | 1. Contaminated TMB. | > Check TMB is colorless or faint blue. |
| | 2. Contaminated reagents (e.g. Conjugate, Wash buffer). | > Check reagents for turbidity. |
| | 3. Incorrect dilution of serum. | > Repeat assay, ensuring correct serum dilution is used. |
| | 4. Incorrect storage of kit. | > Ensure kit is stored at 2-8ºC, plate is sealed in foil pouch and desiccant sachet is blue / purple. |
| | 5. Inefficient washing- Wash buffer left in wells, inconsistent washing, inadequate washing. | > Tap out wash buffer after washing.<br>> Check wells are sufficiently and uniformly filled an aspirated when washing. |
| | 6. If Conjugate reconstitute is required – Conjugate reconstituted incorrectly. | > Repeat assay ensuring Conjugate is reconstituted according to assay method. |

| | | |
|---|---|---|
| All wells negative | 1. Test not performed correctly – correct reagents not added or not added in the correct sequence. | > Check procedure and check for unused reagents.<br>> Ensure that Stop Solution was not added before Conjugate or TMB.<br>> Ensure that serum was diluted in correct Sample diluent; e.g. do not use Sample Absorbent for an IgG ELISA. |
| | 2. Contaminated Conjugate solution. | > Dispense Conjugate directly from the bottle using a clean pipette tip; avoid transferring Conjugate to another container if possible.<br>> Do not return unused Conjugate to bottle.<br>> Ensure all pipettes and probes used to dispense the Conjugate are clean and free from serum, detergent and bleach. |
| | 3. Over- washing of plate (e.g. inclusion of a long soak step). | > Repeat assay using recommended wash procedure. |
| | 4. Incorrect storage of kit. | > Ensure kit is stored at 2-8ºC, plate is sealed in foil pouch and desiccant sachet is blue / purple. |
| | 5. Wash Buffer made up with Stop Solution instead of Wash Buffer Concentrate | > Ensure Wash Buffer is made up correctly. |