HSP60 (human), ELISA kit

For the detection and quantitation of Hsp60 in cell lysates, tissue extracts and serum.

Catalog Number: ADI-EKS-600

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A. INTRODUCTION

ASSAY DESIGN

The HSP60 (human), ELISA kit provides a reliable and sensitive method to detect and quantitate Hsp60 in cell lysates, tissue extracts or serum. The assay is specific for Hsp60 and does not detect other Hsp60 family members, such as GroEL and Hsp65.

HSP60 (human), ELISA is a quantitative sandwich immunoassay. A mouse monoclonal antibody specific for Hsp60 is pre-coated on the wells of the provided Anti-Hsp60 Immunoassay Plate. Hsp60 is captured by the immobilized antibody and is detected with an Hsp60 specific, goat polyclonal antibody. The goat polyclonal antibody is subsequently bound by a horseradish peroxidase conjugated anti-goat IgG secondary antibody. The assay is developed with tetramethylbenzidine substrate and a blue color develops in proportion to the amount of captured Hsp60. 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. Hsp60 concentrations from the sample are quantitated by interpolating absorbance readings from a standard curve generated with the calibrated Hsp60 protein standard provided.

The traditional method for Hsp60 detection and quantitation is accomplished in two steps: immunoblotting followed by densitometry scanning. HSP60 (human), ELISA provides researchers with a rapid and reliable method to measure the levels of Hsp60 in numerous samples. This kit has the potential of expanding our knowledge of Hsp60's role in critical cellular processes or implicating Hsp60 as a diagnostic tool to evaluate and monitor a variety of diseases.

SCIENTIFIC OVERVIEW

Heat shock protein 60 (Hsp60) is a molecular chaperone that participates in the folding and assembly of mitochondrial proteins and facilitates proteolytic degradation of misfolded or denatured proteins. Hsp60 is a member of a highly conserved family which includes molecular chaperones from several species such as plant Hsp60 (also known as Rubisco binding protein), GroEL, the *E. coli* Hsp60, and Hsp65, a major antigen of mycobacteria. Hsp60 is strongly dependent upon its co-chaperone, Hsp10 that binds the molecule and regulates its substrate binding and ATPase activity. Hsp60 is rapidly upregulated by a range of cellular insults including oxidative stress, viral infection, chemical exposure, increased temperature and irradiation[1,2,3,4,5].

Hsp60 is encoded by nuclear DNA and is synthesized as a larger precursor form containing an N-terminal targeting sequence that is necessary for mitochondrial import and is then cleaved to the mature form in the mitochondrial matrix. Hsp60 is mainly located in the mitochondria but changes in the intracellular location and cell surface expression have been reported [5,6,7,8,9,10]. Hsp60 has been detected in the serum of normal individuals and progressive decline in serum Hsp60 levels with aging have been demonstrated. However further research is required to understand the basis of this decline with age and the role of Hsp60 in the maintenance of homeostasis and resistance to environmental challenges[11,12,13,14].

Levels of circulating Hsp60 are associated with early human cardiovascular disease[11,12] and with the presence of physiological distress in women[15]. In addition, there is a significant positive association between serum Hsp60 and TNFα levels [15]. Heat shock proteins have been implicated as antigenic targets for the activation of lesional T cells in atherosclerotic plaques and there is a strong correlation between soluble Hsp60 (sHSP60) and atherosclerosis suggesting that sHsp60 may play important roles in activating vascular cells and the immune system during the development of atherosclerosis[12].

A high prevalence of circulating Hsp60 and Hsp70 has been demonstrated in women with a history of recurrent vulvovaginitis (RV) and current bacterial vaginosis or vaginal candidiasis but not in women with no history of RV, suggesting that differences in induction of heat shock proteins may be related to susceptibility to RV infections[16]. In addition, expression of Hsp60 has been demonstrated in the peritoneal fluids from women with endometriosis indicating that Hsp60 may be released as a consequence of implanted ectopic endometrium[17]. The survival curve analysis of 247 patients with epithelial ovarian carcinoma demonstrated a significant difference in favor of those tumors expressing Hsp60 (median survival 28 versus 37 months), suggesting that Hsp60 expression is associated with a significantly better prognosis[18].

The quantitative measurement of Hsp60 levels in various cell types, tissues and bodily fluids may provide new insights into the physiological roles of Hsp60 in a range of disease states and may lead to the identification of new markers for disease severity and prognosis**.**

ASSAY PROCEDURE SUMMARY

1. Bring to room temperature: **Anti-Hsp60 Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent**, **Antibody Diluent**, **HRP Conjugate Diluent**, **TMB Substrate** and **Stop Solution 2**.
2. Prepare **Recombinant Hsp60 Standard** and samples in **Sample Diluent**.
3. Add 100µL prepared standards and samples in duplicate to wells of **Anti-Hsp60 Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 1 hour.
5. Wash wells 6X with 1X Wash Buffer.
6. Add 100µL diluted **Anti-Hsp60** to each well. Cover immunoassay plate.
7. Incubate plate at room temperature for 1 hour.
8. Wash wells 6X with 1X Wash Buffer.
9. Add 100µL diluted **HRP Conjugate** to each well. Cover immunoassay plate.
10. Incubate plate at room temperature for 30 minutes.
11. Wash wells 6X with 1X Wash Buffer.
12. Add 100µL **TMB Substrate** to each well.
13. Incubate at room temperature for 15 minutes.
14. Add 100µL **Stop Solution 2** to each well.
15. Measure absorbance at 450nm.
16. Plot the Hsp60 standard curve and calculate Hsp60 sample concentrations.

B. MATERIALS

PRECAUTIONS

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- The activity of the **HRP Conjugate** *(part# 80-1525)* is affected by nucleophiles such as azide, cyanide and hydroxylamine.

- The **Stop Solution 2** *(part# 80-0377)* is corrosive; please use caution when handling.

Please read the complete kit insert before performing this assay.

MATERIALS PROVIDED

The HSP60 (human), ELISA kit contains the following components in sufficient quantities for 96 wells. These reagents are sufficient to assay one standard curve and 40 samples in duplicate or two standard curves and 32 samples in duplicate.

PART #	COMPONENT	SIZE	DESCRIPTION
80-1522	Anti-Hsp60 Immunoassay Plate	96 well plate	12 x 8 removable strips and plate frame. Pre-coated plate with mouse monoclonal antibody specific for Hsp60
80-1526	5X Extraction Reagent	10 mL	Concentrated buffer for preparation of cell and tissue extracts
80-1523	Recombinant Hsp60 Standard	25 µL	10µg/mL stock solution of Recombinant Hsp60 protein
80-1760	Sample Diluent	50 mL	Buffer to dilute standards and samples
80-1287	20X Wash Buffer	100 mL	Concentrated solution of buffer and surfactant
80-1524	Anti-Hsp60	25 µL	Goat polyclonal antibody specific for Hsp60
80-1527	Antibody Diluent	11 mL	Buffer for dilution of Anti-Hsp60
80-1525	HRP Conjugate	25 µL	Horseradish peroxidase conjugated to anti-goat IgG
80-1508	HRP Conjugate Diluent	11 mL	Buffer for dilution of HRP Conjugate
80-0350	TMB Substrate	10 mL	Stabilized tetramethylbenzidine substrate
80-0377	Stop Solution 2	10 mL	Acid stop solution to stop color reaction

STORAGE OF MATERIALS

All reagents are stable as supplied at 4°C, except the **Recombinant Hsp60 Standard**, which should be stored at -20°C. For optimum storage, the **Recombinant Hsp60 Standard** should be aliquotted into smaller portions and stored at -20°C.

Unused wells of the **Anti-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 HSP60 (human), ELISA kit contains a pre-coated microtiter plate (**Anti-Hsp60 Immunoassay Plate**) with removable wells to allow assaying on two separate occasions.

- A **5X Extraction Reagent** has been included in this assay. Use of other lysis or extraction buffers may interfere with the performance of the assay.

- Run both standards and samples in duplicate.

- Include a standard curve each time the assay is performed.

- The following kit components should be brought to room temperature prior to use: **Anti-Hsp60 Immunoassay Plate**, **Sample Diluent**, **Wash Buffer**, **Antibody Diluent, HRP Conjugate Diluent, 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.

- For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 15 minutes.

- 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.

PERFORMING THE ASSAY

- 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.

- In this protocol, room temperature refers to 20-28°C. The room temperature should remain within this range throughout the assay.

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

SAMPLE PREPARATION

1. EXTRACTION OF SAMPLES

Suggested protocols for the preparation of cell lysates, tissue extracts and serum samples may be found in Appendices I-III, respectively (pages 23 to 25). Investigators may use alternative methods of cell and tissue lysate preparation, however, it is recommended that the **5X Extraction Reagent** provided in this kit be diluted to 1X and used as the lysis buffer.
Use of alternative lysis buffers may contain components, which could interfere and compromise the performance of the assay, producing inaccurate results. For a complete list of known chemical compatibility within this assay, please refer to Appendix IV (page 26).

2. DILUTION OF SAMPLES

Samples should be prepared as described in Appendix I-III. Cell and tissue lysates may be diluted 1:50 (v/v) in **Sample Diluent** as a suggested starting dilution only. Serum samples may be diluted appropriately in **Sample Diluent** if values for these are not within the range of the standard curve. Users must determine the optimal sample dilutions for their particular experiments.

a) Dilute prepared samples (i.e. cell and tissue lysates, serum) in **Sample Diluent**. Prepare at least 250µL 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 16). Samples may be left at room temperature while Reagents are being prepared (see page 12).

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 8).*

1. TEMPERATURE OF REAGENTS

Bring the following reagents to room temperature prior to use:

- **Anti-Hsp60 Immunoassay Plate** *(Part#: 80-1522)*
- **Sample Diluent** *(Part#: 80-1760)*
- **Wash Buffer** *(Part#: 80-1287)*
- **Antibody Diluent** *(Part#: 80-1527)*
- **HRP Conjugate Diluent** *(Part#: 80-1508)*
- **TMB Substrate** *(Part#: 80-0350)*
- **Stop Solution 2** *(Part#: 80-0377)*

2. RECOMBINANT HSP60 STANDARD *(Part#: 80-1523)*

NOTE*: The Hsp60 Standard will withstand two freeze/thaw cycles to allow preparation of a second standard curve. However, to ensure product integrity, it is suggested that the Hsp60 Standard be aliquotted into smaller portions and any remaining Hsp60 Standard be discarded after the second use.*

The **Hsp60 Standard** is used to generate a standard curve with 6 points, ranging from 3.125 - 100ng/mL.

a) Centrifuge the **Hsp60 Standard** vial before removing the cap. This process will assure that all of the standard is collected and available for use.

b) Label six (6) polypropylene tubes, each with one of the following standard values: 100ng/mL, 50ng/mL, 25ng/mL, 12.5ng/mL, 6.25ng/mL, 3.125ng/mL.

c) Add 500µL of **Sample Diluent** to Tube #1.

d) Add 250µL of **Sample Diluent** to Tube #2, 3, 4, 5, and 6.

e) Add 5µL of the **Hsp60 Standard** stock solution (10µg/mL) to Tube #1.

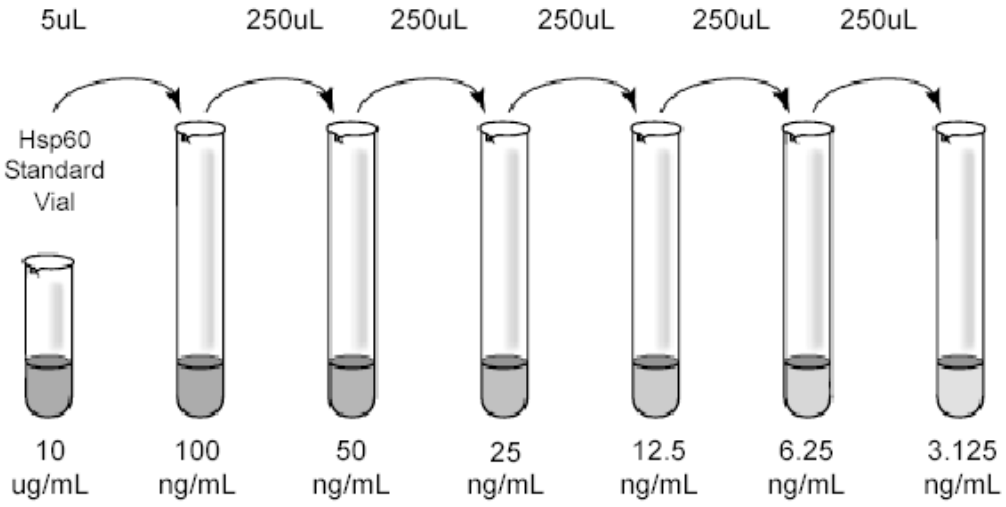
f) Mix thoroughly.

g) Transfer 250µL from Tube#1 to Tube #2.

h) Mix thoroughly.

i) Similarly, complete the dilution series to generate the remaining standards (250µL from Tube #2 to Tube #3, mix thoroughly, etc) up to and including Tube #6.

j) Finally, add 250µL **Sample Diluent** to another 1.5mL polypropylene tube (Tube # 7), which is the zero standard (0ng/mL).



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 100 mL of **20X Wash Buffer** with 1900 mL 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 520mL (if the complete plate is used at once). However additional 1X Wash Buffer is supplied to allow for multiple assays or alternative washing techniques.*

4. ANTI-HSP60 *(Part#: 80-1524)*

   a) Centrifuge the vial before removing the cap to ensure maximum product recovery.

   b) Dilute 22µL of **Anti-Hsp60** in 11mL of **Antibody Diluent** in a polypropylene tube. If only using a portion of the plate, dilute only what is needed for number of wells used.

   c) Mix gently by inversion.

   d) Reagent is now ready to be used in the Assay Procedure (see page 15).

   e) Do not re-use or store any remaining diluted **Anti-Hsp60**.

5. HRP CONJUGATE *(Part#: 80-1525)*

   a) Centrifuge the vial before removing the cap to ensure maximum product recovery.
   b) Dilute 22µL of the **HRP Conjugate** in 11mL of the **HRP Conjugate Diluent** in a polypropylene tube. If only using a portion of the plate, dilute only what is needed for the number of wells used.
   c) Mix gently by inversion.
   d) Reagent is now ready to be used in the Assay Procedure (see below).
   e) Do not re-use or store any remaining diluted **HRP Conjugate**.

ASSAY PROCEDURE

1. DETERMINE THE REQUIRED NUMBER OF WELLS

   a) Refer to the Hsp60 Plate Template on page 27 to determine the number of wells to be used.
   b) Remove the **Anti-Hsp60 Immunoassay Plate** from the packaging and note the color of the desiccant pack. Silica beads should be blue. Pink beads indicate that moisture is present and the performance of the plate may be compromised.
   c) If less than 96 pre-coated microtiter wells are needed, remove the excess wells from the frame and return them to the foil pouch.
   d) Reseal the pouch containing the unused wells and store at 4°C.

2. ADDITION OF STANDARDS AND SAMPLES

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

- Prepared **Hsp60 Standard** (Tube#1 through Tube #6)
- Samples (previously prepared - see Sample Preparation, page 11)
- Zero Standard (**Sample Diluent**, which represents 0ng/mL)

b) Cover wells with an adhesive plate sealer or plastic wrap and incubate at room temperature for 1 hour.

***NOTE**: For each step in the procedure, total dispensing time for the addition of the reagents and samples to the assay plate should not exceed 15 minutes.*

3. WASHING

a) Aspirate liquid from all wells.

b) Add 300µL of 1X Wash Buffer to all wells, using a multi-channel pipette, manifold dispenser, automated microplate washer, or a squirt bottle.

c) Repeat the aspirating and washing 5 more times, for a total of 6 washes.

d) After the 6th 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-HSP60 ANTIBODY

(*previously diluted, see page 14)*

a) Add 100µL of the previously diluted **Anti-Hsp60** to each well, except the blank.

b) Cover wells with a fresh adhesive plate sealer (or plastic wrap) and incubate at room temperature for 1 hour .

c) Wash plate as described in Step #3.

5. ADDITION OF HRP CONJUGATE
(previously diluted, see page 15)

a) Add 100µL of the previously diluted **HRP Conjugate** to each well, except the blank.
b) Cover wells with a fresh adhesive plate sealer (or plastic wrap) and incubate at room temperature for 30 minutes.
c) Wash plate as described in Step #3.

6. ADDITION OF TMB SUBSTRATE AND STOP SOLUTION

a) Add 100µL of the **TMB Substrate** to every well. Color development should be visible within 1 minute of addition to the plate.
b) Incubate the plate at room temperature for 15 minutes.
c) Add 100µL of the **Stop Solution 2** to every well in the same order that the **TMB Substrate** was added.

7. MEASURING ABSORBANCE

a) Set up the microplate reader according to the manufacturer's instructions.
b) Set wavelength at 450nm.
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 2**.

CALCULATION OF RESULTS - DETERMINATION OF 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 blank.

3. Subtract the average value obtained in Step#2 (blank) from the values obtained in Step#1 (standards and samples).

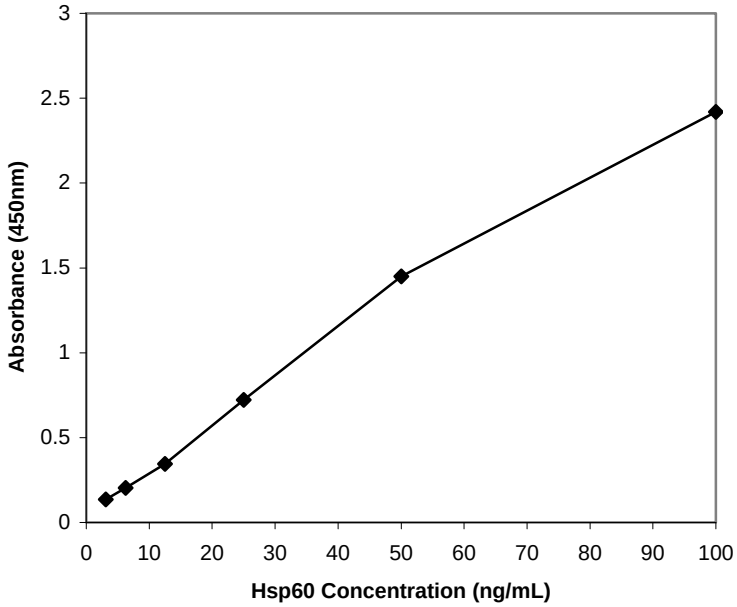
4. To generate the standard curve, plot the Recombinant Hsp60 Standard concentrations (ng/mL) on theX-axis, and the absorbance measurements for the corresponding Hsp60 standards on the Y-axis. Determine the best fit line.

5. Interpolate the sample concentrations from the standard curve and multiply by the dilution factor for the final sample 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 Hsp60 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 HSP60 STANDARD CURVE



PERFORMANCE CHARACTERISTICS

1. SENSITIVITY

The sensitivity of the HSP60 (human), ELISA has been determined to be 3.125ng/mL.
The standard curve has a range of 3.125 - 100ng/mL.

2. PRECISION

a) Intra-Assay Precision (Within Run Precision)
To determine Intra-Assay Precision, three samples of known concentration were assayed thirty times on one plate.
The Intra-Assay Coefficient of variation of HSP60 (human), ELISA has been determined to be <10%.

b) Inter-Assay Precision (Between Run Precision)
To determine Inter-Assay Precision, three samples of known concentration were assayed thirty times in three individual assays. The Inter-Assay Coefficient of variation of the Hsp60 ELISA has been determined to be <10%.

3. SPECIFICITY AND SPECIES REACTIVITY

HSP60 (human), ELISA is specific for Hsp60. The assay does not cross react with 100ng/mL of GroEL, Hsp65, Hsp27, Hsp25, Cpn10, or GroES. The Hsp60 ELISA has been certified for the detection of human Hsp60.

LIMITATIONS OF THE ASSAY

- This assay has been validated for use with cell lysates, tissue extracts and serum. Other sample types or matrices (e.g. urine, cerebrospinal fluid, cell culture supernatant, etc.) may contain interfering factors that can compromise the performance of the assay, or produce inaccurate results.

- Although this assay has been validated for use with cell lysates, tissue extracts and serum, some samples may contain higher levels of interfering factors that can produce anomalous results.

- If 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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F. APPENDICES

APPENDIX I
PREPARATION OF CELL LYSATES

1. For adherent cell lines, aspirate the media and wash the cells three times with phosphate buffered saline. Harvest the cells using appropriate, established methods (e.g. scraping, trypsinization) and centrifuge cells to pellet.
2. For non-adherent cell lines, centrifuge cells to pellet, aspirate media and wash cells three times with phosphate buffered saline.
3. Aspirate the supernatant from the final wash.
4. If necessary, the cell pellet can be frozen at -70°C and processed at a later date.
5. Calculate the amount of 1X Extraction Reagent that will be required. For every 1 X 10^6 to 1 X 10^7 cells, use 1ml of 1X Extraction Reagent.
6. Dilute an appropriate amount of 5X Extraction Reagent with cold (4°C) deionized or distilled water to generate the required volume of 1X Extraction Reagent. For example, if 5ml of 1X Extraction Reagent were required, dilute 1ml of the 5X Extraction Reagent with 4ml of cold deionized or distilled water.
7. Add protease inhibitors to the 1X Extraction Reagent. Examples of appropriate protease inhibitors include 0.1mM PMSF, 1µg/ml leupeptin, 1µg/ml aprotinin, 1µg/ml pepstatin. Alternatively, a protease inhibitor cocktail tablet can be added at a final 1X concentration. Protease inhibitor cocktail tablets are commercially available from a variety of scientific reagent vendors.
8. Resuspend the cell pellet with an appropriate volume of 1X Extraction Reagent supplemented with protease inhibitors. Pipet up and down to break up the cell pellet until the cell suspension is homogeneous and no clumps are visible.
9. Incubate 30 minutes on ice with occasional mixing or alternatively, samples can be briefly sonicated.
10. Transfer extracts to polypropylene microcentrifuge tubes and centrifuge at 21,000 x g for 10 minutes in a 4°C refrigerated microfuge.
11. Transfer the supernatants to labeled polypropylene tubes. When collecting the supernatant, avoid disturbing the cell pellet. The supernatant collected is the cell lysate, which is now ready for analysis using the Hsp60 ELISA kit. The resulting pellets can be discarded.
12. Alternatively, the cell lysates can be frozen at -70°C and assayed at a later date. It is recommended that a protein assay be performed and the lysates aliquotted to convenient amounts prior to storing at -70°C to avoid multiple freeze thaw cycles.

APPENDIX II
PREPARATION OF TISSUE EXTRACTS

1. Harvest tissue to be analyzed.
2. If necessary, tissues can be flash frozen, stored at -70°C and the extract prepared at a later time.
3. Calculate the amount of 1X Extraction Reagent that will be required. For each ~0.5cm^3 piece of tissue, use 1ml of 1X Extraction Reagent.
4. Dilute an appropriate amount of 5X Extraction Reagent with cold (4°C) deionized or distilled water to generate the required volume of 1X Extraction Reagent. For example, if 5ml of 1X Extraction Reagent were required, dilute 1ml of the 5X Extraction Reagent with 4ml of cold deionized or distilled water.
5. Add protease inhibitors to the 1X Extraction Reagent. Examples of appropriate protease inhibitors include 0.1mM PMSF, 1µg/ml leupeptin, 1µg/ml aprotinin, 1µg/ml pepstatin. Alternatively, a protease inhibitor cocktail tablet can be added at a final 1X concentration. Protease inhibitor cocktail tablets are commercially available from a variety of scientific reagent vendors.
6. Place the tissue in a mortar and add a sufficient volume of liquid nitrogen to cover the tissue.
7. Allow the liquid nitrogen to evaporate. The tissue should be thoroughly frozen.
8. Grind the frozen tissue to a powder with a pestle.
9. Add an appropriate volume of 1X Extraction Reagent supplemented with protease inhibitors to the processed tissue.
10. Continue to homogenize the tissue with the pestle until the tissue suspension is homogeneous.
11. Transfer the extract to a polypropylene tube and centrifuge at 21,000 x g for 10 minutes in a 4°C refrigerated microfuge.
12. Transfer the supernatant to a labeled polypropylene tube. The supernatant collected is the tissue extract, which is now ready for analysis using the Hsp60 ELISA kit. The resulting pellet can be discarded.
13. Alternatively, the tissue extracts can be frozen at -70°C and assayed at a later date. It is recommended that a protein determination assay be performed and the extracts aliquotted to convenient amounts prior to storing at -70°C to avoid multiple freeze thaw cycles.

APPENDIX III
COLLECTION OF SERUM

1. Collect whole blood using established methods.
2. Allow samples to clot at room temperature for 30 minutes.
3. Centrifuge at 2700 x g for 10 minutes, taking precautions to avoid hemolysis.
4. Remove serum. Transfer the serum to a labeled polypropylene tube. The serum collected is now ready for analysis using the Hsp60 ELISA kit.
5. 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.

APPENDIX IV
CHEMICAL COMPATIBILITY LIMITS

Different chemicals may interfere with the Hsp60 ELISA kit. Although the effect of every chemical is not known, Enzo Life Sciences has tested the following chemicals to determine the level at which they may interfere with the kit.
The compatible limit is defined as the chemical concentration at which the measurement of Hsp60 in a sample is inhibited by ≤10%.

CHEMICAL	COMPATIBLE LIMIT
Aprotinin	50µg/mL
ß-mercaptoethanol	0.15mM
CHAPS	0.05% (w/v)
Dithiothreitol (DTT)	0.2mM
EDTA	100mM
Glycerol	1% (v/v)
HEPES, pH 7.5	25mM
Leupeptin	50µg/mL
Magnesium Chloride (MgCl2)	50mM
MOPS, pH 7.5	250mM
NP-40	0.05% (v/v)
Pepstatin A	10µg/mL
PMSF	5mM
SDS	0.01% (w/v)
Sodium Azide (NaN3)	0.5% (w/v)
Sodium Deoxycholate	0.2% (w/v)
Sodium Chloride (NaCl)	150mM
Sodium Phosphate, pH 7.2	15mM
Tris, pH 7.5	5mM
Triton-X100	0.01% (v/v)
Tween-20	<0.005% (v/v)

APPENDIX V - Hsp60 Immunoassay Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	12.5ng/mL										
B	Blank	12.5ng/mL										
C	100ng/mL	6.25ng/mL										
D	100ng/mL	6.25ng/mL										
E	50ng/mL	3.125ng/mL										
F	50ng/mL	3.125ng/mL										
G	25ng/mL	0ng/mL										
H	25ng/mL	0ng/mL										

NOTES

REFERENCE

1. Bring to room temperature: **Anti-Hsp60 Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent**, **Antibody Diluent**, **HRP Conjugate Diluent**, **TMB Substrate** and **Stop Solution 2**.
2. Prepare **Recombinant Hsp60 Standard** and samples in **Sample Diluent**.
3. Add 100µL prepared standards and samples in duplicate to wells of **Anti-Hsp60 Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 1 hour.
5. Wash wells 6X with 1X Wash Buffer.
6. Add 100µL diluted **Anti-Hsp60** to each well. Cover immunoassay plate.
7. Incubate plate at room temperature for 1 hour.
8. Wash wells 6X with 1X Wash Buffer.
9. Add 100µL diluted **HRP Conjugate** to each well. Cover immunoassay plate.
10. Incubate plate at room temperature for 30 minutes.
11. Wash wells 6X with 1X Wash Buffer.
12. Add 100µL **TMB Substrate** to each well.
13. Incubate at room temperature for 15 minutes.
14. Add 100µL **Stop Solution 2** to each well.
15. Measure absorbance at 450nm.
16. Plot the Hsp60 standard curve and calculate 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

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