HSP90α (human), ELISA kit

For the detection and quantitative measurement of Hsp90α in biological samples.

Catalog Number: ADI-EKS-895

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

TABLE OF CONTENTS

A. INTRODUCTION

The Hsp90α ELISA Kit provides a sensitive, rapid and reliable method to detect and quantitate Hsp90α in cell lysates, tissue extracts and serum samples from human origin. The Hsp90α ELISA is specific for Hsp90α and does not cross react with Hsp90β, Grp94, Hsp60 and Hsp70.

The Hsp90α ELISA is a quantitative sandwich enzyme-linked immunosorbent assay. The assay uses an Hsp90α specific mouse monoclonal antibody pre-coated on the wells of the provided Anti-Hsp90α Immunoassay Plate to capture Hsp90α in the sample or standard. The captured protein is then detected using an Hsp90α antibody conjugated to horseradish peroxidase. The assay is developed with tetramethylbenzidine (TMB) substrate and a blue color develops in proportion to the amount of captured Hsp90α. 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.

SCIENTIFIC OVERVIEW

The Hsp90 family of proteins are highly conserved and found in all species (1). In unstressed cells, Hsp90 can account for 1-2% of the total cellular protein and this level increases when cells are exposed to heat shock or other stressors (1). Under heat shock conditions, high levels of Hsp90 are required for efficient reactivation of heat-denatured proteins, whereas under normal conditions Hsp90 is required by a specific group of substrate proteins (2). Mammalian Hsp90 generally exists as dimers and share many functional similarities with Hsp70 (1). Other chaperones are frequently found in the Hsp90-complex in the cytoplasm and forms an integral part of a ubiquitous multicomponent-chaperone system (1). Hsp90 is involved in many signal transduction pathways, including glucocorticoid receptor (3), progesterone receptor (4), estrogen receptor (5), dioxin receptor (6), and protein kinases (7,8).

Members of the Hsp90 family are highly conserved in the N-terminal and C-terminal regions (9). Studies have shown that these terminal regions have independent chaperone functions with different substrate specificities (10). The C-terminal has the ATP-independent chaperone activity for substrates,

binding to partially folded polypeptides in an ATP-independent manner (10). The N-terminal domain binds to peptides longer than 10 amino acids, ATP and to the selective Hsp90 inhibitor, Geldanamycin (11).

It was proposed that isoforms in many important genes are a result of gene duplication events, which occurred hundreds of millions of years ago during vertebrate evolution (12). In mouse embryos, the two isoforms Hsp86 and Hsp84 show a differential pattern of expression in the somatic and germ cell compartments of the developing testis (13). Heat shock induces both forms in mouse and human cells (14), whereas in chicken and quail cell lines only Hsp90α, but not Hsp90β is induced by heat shock (15). These studies have concluded that the Hsp90β gene may have acquired heat-inducibility sometime during late vertebrate evolution (15). Zebra fish Hsp90α and Hsp90β exhibit differential pattern of regulation during cell differentiation and development (16). These isoforms even show differences in inducibility in response to heat shock and other stressors (16). Zebra fish Hsp90β was found to be the constitutive form which is weakly inducible, whereas Hsp90α, the inducible form, is highly expressed during developmental stages of the embryo (17). A detailed analysis of the expression patterns strongly suggest that the Hsp90α gene plays a specific role in the normal process of myogenesis, in addition to protecting embryonic cells from environmental stress (18). The different expression profiles of Hsp90 isoforms further emphasizes its functional significance during ontogeny and stress in animals.

Newer studies are even beginning to report the extracellular roles of Hsp90α. Hsp90α was known to act as a chaperone for matrix mettaloproteinase-2 (MMP2) in HT-1080 fibrosarcoma cells, leading to increased tumor invasiveness (19). In recent clinical studies, Hsp90 chaperone complex has been used as a molecular target in the human oesophageal cancer (20). Many studies have identified chaperones as facilitators of malignant transformation at the molecular level, and Hsp90 as a novel target for anticancer drug action (21-23). Geldanamycin has been used to study the cellular role of Hsp90 in childhood tumors (21). Studies with preclinical breast cancer models have used Hsp90-inhibitors in combination with conventional chemotherapy to validate Hsp90 as an oncogenic target in breast cancer (24).

ASSAY PROCEDURE SUMMARY

1. Bring to room temperature: **Anti-Hsp90α Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent**, **HRP Conjugate Diluent**, **TMB Substrate** and **Stop Solution 2**.
2. Prepare **Recombinant Hsp90α Standard** and samples in **Sample Diluent**.
3. Add 100 µL prepared standards and samples in duplicate to wells of **Anti-Hsp90α Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 1 hour.
5. Wash wells 6X with 1X Wash Buffer.
6. Add 100 µL diluted **HRP Conjugate** 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 100 µL **TMB Substrate** to each well.
10. Incubate at room temperature for 20 minutes.
11. Add 100 µL **Stop Solution 2** to each well.
12. Measure absorbance at 450nm.
13. Plot the Hsp90**α** standard curve and calculate Hsp90**α** sample concentrations.

B. MATERIALS

PRECAUTIONS

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- The **Stop Solution 2** *(part# 80-0377)* is a 1N solution of hydrochloric acid. This solution is corrosive; please use caution when handling.

- The activity of the **HRP conjugate** *(part# 80-1561)* is affected by nucleophiles such as azide, cyanide and hydroxylamine.

Please read the complete kit insert before performing this assay.

MATERIALS PROVIDED

The Hsp90α ELISA Kit contains the following components in sufficient quantities for 96 wells. These reagents are sufficient to assay one standard curve and 39 samples in duplicate or two standard curves and 30 samples in duplicate.

PART #	COMPONENT	SIZE	DESCRIPTION
80-1555	Anti-Hsp90α Immunoassay Plate	96 well plate	12 x 8 removable strips and plate frame. Pre-coated plate with Hsp90α monoclonal antibody
80-1526	5X Extraction Reagent	10 mL	Concentrated buffer for preparation of cell and tissue extracts
80-1564	Recombinant Hsp90α Standard	25 µL	1µg/mL stock solution of Recombinant Human Hsp90α 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
80-1561	HRP Conjugate	30 µL	Hsp90α monoclonal antibody conjugated to horseradish peroxidase
80-1563	HRP Conjugate Diluent	11 mL	Buffer for dilution of Anti-Hsp90α: 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 Hsp90α Standard**, which should be stored at -20°C. For optimum storage, the **Recombinant Hsp90α Standard** should be aliquotted into smaller portions and stored at -20°C. Avoid repeated freeze thaw cycles.

Unused wells of the **Anti-Hsp90α Immunoassay Plate** should be resealed with desiccant 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 1000 µL
- Disposable pipette tips
- 5, 10, 25 mL 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 Hsp90α ELISA kit contains a pre-coated microtiter plate (**Anti-Hsp90α Immunoassay Plate**) with removable wells to allow assaying on 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 or triplicate.
- Include a standard curve each time the assay is performed.
- The following kit components should be brought room temperature prior to use: **Anti-Hsp90α Immunoassay Plate**, **Sample Diluent**, **Wash Buffer**, **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.
- 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 19 to 21). 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 22).

2. DILUTION OF SAMPLES

Samples should be prepared as described in Appendix I-III. For un-induced cell and tissue lysates, use 500 µg/mL (total protein concentration) in **Sample Diluent** as a suggested starting dilution only. For induced cell and tissue lysates, use 500 ng/mL (total protein concentration) in **Sample Diluent** as a suggested starting dilution only. Serum samples may be diluted 1:25 (v/v) appropriately in **Sample Diluent** as a suggested starting dilution. 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 250 µL of diluted sample to permit assaying in duplicate (or an appropriate volume if assaying in triplicate).
b) Mix thoroughly.
c) Samples are now ready to be used in the Assay Procedure (see page 13). Samples may be left at room temperature while Reagents are being prepared (see page 10).

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:

- **Anti-Hsp90α Immunoassay Plate** *(Part#: 80-1555)*
- **Sample Diluent** *(Part#: 80-1760)*
- **Wash Buffer** *(Part#: 80-1287)*
- **HRP Conjugate Diluent** *(Part#: 80-1563)*
- **TMB Substrate** *(Part#: 80-0350)*
- **Stop Solution 2** *(Part#: 80-0377)*

2. RECOMBINANT HSP90α STANDARD *(Part#: 80-1564)*

NOTE*: The Hsp90α 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 Hsp90α Standard be aliquotted into smaller portions and any remaining Hsp90α Standard be discarded after the second use.*

The **Hsp90α Standard** is used to generate a standard curve with 7 points, ranging from 0.0625 – 4 ng/mL.

a) Centrifuge the **Hsp90α Standard** vial before removing the cap. This process will assure that all of the standard is collected and available for use.
b) Label seven (7) polypropylene tubes, each with one of the following standard values: 4 ng/mL, 2 ng/mL, 1 ng/mL, 0.5 ng/mL, 0.25 ng/mL, 0.125 ng/mL, 0.0625 ng/mL.

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

d) Add 250 µL of **Sample Diluent** to Tube #2, 3, 4, 5, 6 and 7.

e) Add 2 µL of the **Hsp90α Standard** stock solution (1 µg/mL) to Tube #1.

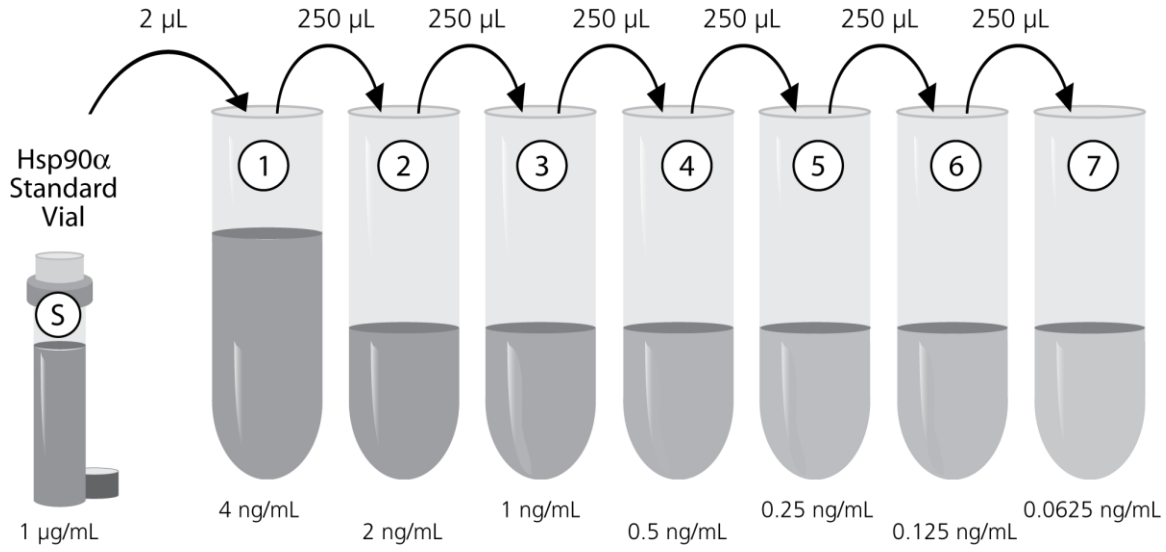
f) Mix thoroughly.

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

h) Mix thoroughly.

i) Similarly, complete the dilution series to generate the remaining standards (250 µL from Tube #2 to Tube #3, mix thoroughly, etc) up to and including Tube #7.

j) Finally, add 250 µL **Sample Diluent** to another 1.5 mL polypropylene tube (Tube # 8), which is the zero standard (0 ng/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**: 100 mL 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 350 mL (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. HRP CONJUGATE *(Part#: 80-1561)*

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

b) Dilute 27.5 µL of **HRP Conjugate** in 11 mL of **HRP Conjugate 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 13).*

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 Hsp90α Plate Template on page 23 to determine the number of wells to be used.
   b) Remove the **Anti-Hsp90α 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 100 µL (in duplicate or triplicate) of each of the following to appropriate wells:
      - Prepared **Hsp90α Standard** (Tube#1 through Tube #7)
      - Samples (previously prepared - see Sample Preparation, page 9)
      - Zero Standard (**Sample Diluent**, which represents 0 ng/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 300 µL 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 HRP CONJUGATE
(*previously diluted, see page 12)*

a) Add 100 µL 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 1 hour.
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 every well. Color development should be visible within 2 minutes of addition to the plate.
b) Incubate the plate at room temperature for 20 minutes.
c) Add 100 µL of the **Stop Solution 2** to every 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.
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 HSP90α CONCENTRATIONS

1. Calculate the average of the duplicate or triplicate absorbance measurements for each standard and sample.

2. Calculate the average of the duplicate or triplicate 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).

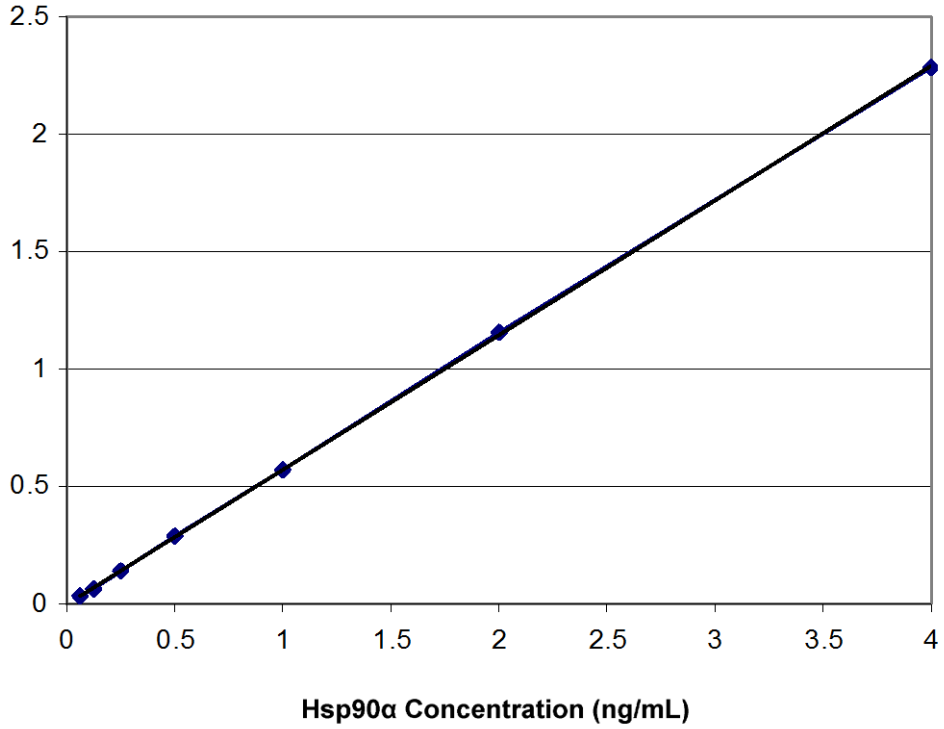
4. To generate the standard curve, plot the Recombinant Hsp90α Standard concentrations (ng/mL) on theX-axis, and the absorbance measurements for the corresponding Hsp90α standards on the Y-axis. Determine the best fit line.

5. Interpolate the sample concentrations from the standard curve and multiply by the dilution factor for the final sample Hsp90α 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 Hsp90α 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 HSP90α STANDARD CURVE



PERFORMANCE CHARACTERISTICS

1. SENSITIVITY

   The sensitivity of the Hsp90α ELISA has been determined to be 50pg/mL. The standard curve has a range of 0 – 4 ng/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 the Hsp90α 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 Hsp90α ELISA has been determined to be <10%.

3. SPECIFICITY AND SPECIES REACTIVITY

The Hsp90α ELISA is specific for Hsp90α and will not react with Hsp90β. The assay does not cross react with 100 ng/mL of Grp94, Hsp60, Hsp70. The Hsp90α ELISA has been certified for the detection of human Hsp90α.

LIMITATIONS OF THE ASSAY

- This assay has been validated for use with cell lysate, 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

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 1 mL 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 5 mL of 1X Extraction Reagent were required, dilute 1 mL of the 5X Extraction Reagent with 4 mL of cold deionized or distilled water.
7. Add protease inhibitors to the 1X Extraction Reagent. Examples of appropriate protease inhibitors include 0.1 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/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 Hsp90αELISA 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 1 mL 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 5 mL of 1X Extraction Reagent were required, dilute 1 mL of the 5X Extraction Reagent with 4 mL of cold deionized or distilled water.
5. Add protease inhibitors to the 1X Extraction Reagent. Examples of appropriate protease inhibitors include 0.1 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/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 Hsp90α 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 Hsp90α 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 Hsp90α 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 Hsp90α in a sample is inhibited by ≤10%.

CHEMICAL	COMPATIBLE LIMIT
Aprotinin	50 µg/mL
ß-mercaptoethanol	0.75 mM
CHAPS	0.5% (w/v)
Dithiothreitol (DTT)	1 mM
EDTA	5 mM
Glycerol	1% (v/v)
HEPES, pH 7.5	5 mM
Leupeptin	1 µg/mL
Magnesium Chloride (MgCl2)	50 mM
MOPS, pH 7.5	50 mM
NP-40	5% (v/v)
Pepstatin A	50 µg/mL
PMSF	1 mM
SDS	0.01%
Sodium Azide (NaN3)	0.05% (w/v)
Sodium Deoxycholate	0.005% (w/v)
Sodium Chloride (NaCl)	250 mM
Sodium Phosphate, pH 7.2	25 mM
Tris, pH 7.5	25 mM
Triton-X100	5% (v/v)
Tween-20	5% (v/v)

APPENDIX V - Hsp90α Immunoassay Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	0.5 ng/mL	0 ng/mL									
B	Blank	0.5 ng/mL	0 ng/mL									
C	4 ng/mL	0.25 ng/mL										
D	4 ng/mL	0.25 ng/mL										
E	2 ng/mL	0.125 ng/mL										
F	2 ng/mL	0.125 ng/mL										
G	1 ng/mL	0.0625 ng/mL										
H	1 ng/mL	0.0625 ng/mL										

Notes

Notes

REFERENCE

1. Bring to room temperature: **Anti-Hsp90α Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent**, **HRP Conjugate Diluent**, **TMB Substrate** and **Stop Solution 2**.
2. Prepare **Recombinant Hsp90α Standard** and samples in **Sample Diluent**.
3. Add 100 µL prepared standards and samples in duplicate to wells of **Anti-Hsp90α Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 1 hour.
5. Wash wells 6X with 1X Wash Buffer.
6. Add 100 µL diluted **HRP Conjugate** 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 100 µL **TMB Substrate** to each well.
10. Incubate at room temperature for 20 minutes.
11. Add 100 µL **Stop Solution 2** to each well.
12. Measure absorbance at 450nm.
13. Plot the Hsp90α standard curve and calculate Hsp90α sample concentrations.

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