ELISA PRODUCT INFORMATION & MANUAL

HspA6

NBP2-62153

Enzyme-linked Immunosorbent Assay for quantitative detection of Human HspA6.
For research use only.
Not for diagnostic or therapeutic procedures.

www.novusbio.com - P: 303.730.1950 - P: 888.506.6887 - F: 303.730.1966 - technical@novusbio.com

Novus kits are guaranteed for 6 months from date of receipt

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A. INTRODUCTION

ASSAY DESIGN

The HSPA6 (HspA6) EIA kit provides a sensitive and specific method to detect and quantitate HspA6 in cell lysates, tissue extracts and serum samples from human origin. The assay is specific for HspA6 and does not detect other Hsp70 family members, such as Hsp70, Hsc70, Hsp71 *(M. tuberculosis)*, Grp78, or DnaK (*E. coli*).

This is a quantitative sandwich immunoassay. A mouse monoclonal antibody specific for HspA6 is pre-coated on the wells of the provided Anti-HspA6 Immunoassay Plate. HspA6 is captured by the immobilized antibody and is detected with an HspA6 specific, rabbit polyclonal antibody. The polyclonal antibody is subsequently bound by a horseradish peroxidase conjugated anti- rabbit IgG secondary antibody. The assay is developed with tetramethylbenzidine substrate and a blue color develops in proportion to the amount of captured HspA6. 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 450 nm. HspA6 concentrations from the sample are quantitated by interpolating absorbance readings from a standard curve generated with the calibrated HspA6 protein standard provided.

SCIENTIFIC OVERVIEW

The Hsp70 system is a highly-conserved family of ubiquitous proteins found in all prokaryotes and in cellular compartments of eukaryotic organisms (1).
Multiple members of this family are involved in protein folding and several other cellular functions (2). Some forms are constitutively expressed in cells, while others are only inducible by metabolic stress (2). Various stressors that induce Hsp70 include heat shock, hypoxia, UV irradiation, $CdCl_2$ and arsenite (1,3). Basal levels of constitutive Hsp70 are found in major intracellular compartments and the inducible Hsp70s are predominantly cytoplasmic and nuclear in distribution (2). There are at least 11 genes for proteins of the human Hsp70 family, which code for a group of highly related proteins ranging from 66 to 78 kDa (2). The multiple members of this family vary with their basal expression levels and inducibility in response to different stressors (2). The Hsp70 chaperones have two major functional domains (4). The highly conserved NH2-terminal domain has ATPase activity and binds to ADP and ATP very tightly, and COOH-terminal binds to polypeptides (4). Hsp70 is known to bind preferentially to unfolded and partially folded proteins and prevent their aggregation or misfolding (2). The nomenclature of the different members of Hsp70 family is extensive and is based on cellular distribution and inducibility (refer to Appendix VI, page 24, for nomenclature of Hsp70 family members) (2).

Human HspA6 is a variant Hsp70 that is more basic than the major Hsp70 and has different stress-induction characteristics (5,6). There have been several reports of Hsp70 variants, but most of them are products of different postranslational modifications (5). HspA6 is a product of a separate gene which is devoid of introns similar to Hsp70 (5). The HspA6 gene has 77% sequence identity to Hsp70 gene and 70% identity to Hsc70 cDNA with highest sequence divergence at the 3'- end (5). Promoter studies have shown HspA6 to be a unique member of the Hsp70 family (5). Unlike Hsp70 which shows basal levels of expression and is induced by heat and various stressors, HspA6 is strictly heat-inducible and shows no basal levels (5). One study reported the use of HspA6 as a target gene for studying single nucleotide polymorphisms (SNP) in biopsy samples of human prostate cancer patients (7).

A. INTRODUCTION

ASSAY PROCEDURE SUMMARY

1. Bring to room temperature: **Anti-HspA6 Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent**, **Anti-HspA6 Diluent, HRP Conjugate Diluent**, **TMB Substrate** and **Stop Solution 2**.
2. Prepare **Recombinant HspA6 Standard** and samples in **Sample Diluent**.
3. Add 100µL prepared standards and samples in duplicate to wells of **Anti- HspA6 Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 2 hours.
5. Wash wells 6X with 1X Wash Buffer.
6. Add 100µL diluted **Anti-HspA6** 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 1 hour.
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 450 nm.
16. Plot the HspA6 standard curve and calculate HspA6 sample concentrations.

B. MATERIALS

PRECAUTIONS

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

- The **Stop Solution 2** is a solution of acid. This solution is corrosive; please use caution when handling.

- The activity of the **HRP Conjugate** is affected by nucleophiles such as azide, cyanide and hydroxylamine.

Please read the complete kit insert before performing this assay.

MATERIALS PROVIDED

The HspA6 EIA 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.

COMPONENT	SIZE	DESCRIPTION
Anti-HspA6 Immunoassay Plate	96 well plate	12 x 8 removable strips and plate frame. Pre-coated plate with HspA6 monoclonal antibody
5X HspA6 Extraction Reagent	10 mL	Concentrated buffer for preparation of cell and tissue extracts
Recombinant HspA6 Standard	25 µL	1µg/mL stock solution of recombinant human HspA6 protein
Sample Diluent	50 mL	Buffer to dilute standards and samples
20X Wash Buffer	100 mL	Concentrated solution of buffer and surfactant
Anti-HspA6	25 µL	Rabbit polyclonal antibody specific for HspA6
Anti-HspA6 Diluent	11 mL	Buffer for dilution of Anti-HspA6
HRP Conjugate	25 µL	Horseradish peroxidase conjugated to anti-rabbit IgG
HRP Conjugate Diluent	11 mL	Buffer for dilution of HRP Conjugate
TMB Substrate	10 mL	Stabilized tetramethylbenzidine substrate
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 HspA6 Standard**, which should be stored at -20°C. For optimum storage, the **Recombinant HspA6 Standard** should be aliquotted into smaller portions and stored at -20°C. Avoid repeated freeze thaw cycles.

Unused wells of the **Anti-HspA6 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 450 nm
- Adhesive plate sealers or plastic wrap

C. PERFORMING THE ASSAY

CRITICAL ASSAY PARAMETERS AND NOTES

- The HspA6 EIA kit contains a pre-coated microtiter plate (**Anti-HspA6 Immunoassay Plate**) with removable wells to allow assaying on separate occasions.
- A **5X HspA6 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 room temperature prior to use: **Anti-HspA6 Immunoassay Plate**, **Sample Diluent**, **Wash Buffer**, **Anti-HspA6 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.
- 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.
Investigators may use alternative methods of cell and tissue lysate preparation; however, it is recommended that the **5X Hsp70B' 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 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 1:20 (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.
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-HspA6 Immunoassay Plate**
- **Sample Diluent**
- **Wash Buffer**
- **Anti-HspA6 Diluent**
- **HRP Conjugate Diluent**
- **TMB Substrate**
- **Stop Solution 2**

2. RECOMBINANT HSPA6 STANDARD

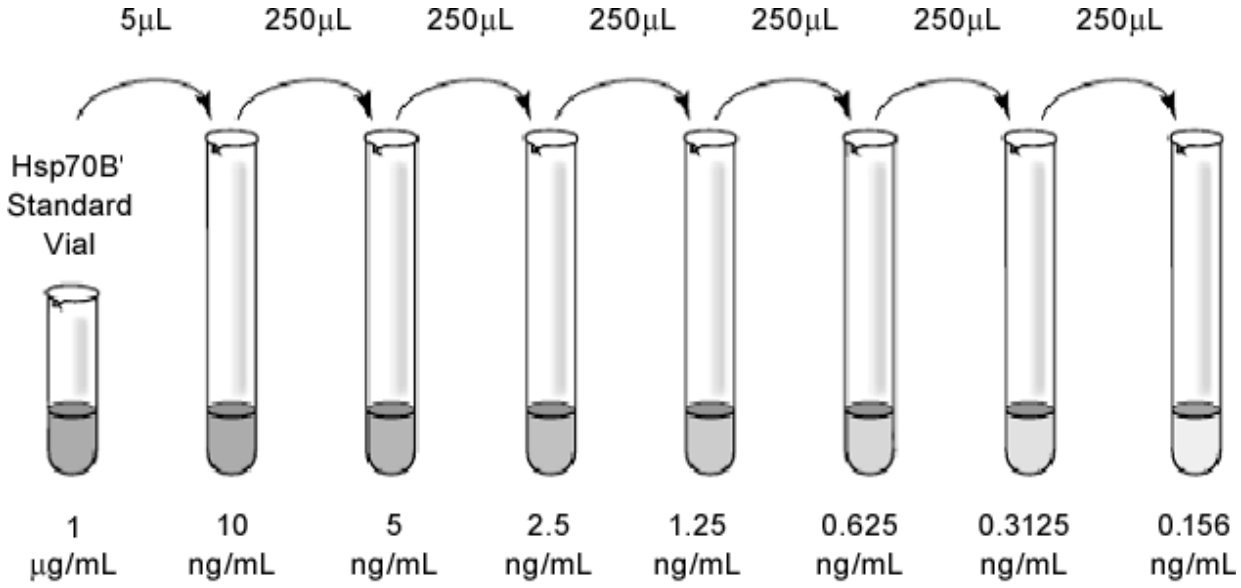
NOTE*: The HspA6 Standard will withstand two freeze/thaw cycles to allow preparation of a second standard curve. However, to ensure product integrity, it is suggested that the HspA6 Standard be aliquotted into smaller portions and any remaining HspA6 Standard be discarded after the second use.*

The **HspA6 Standard** is used to generate a standard curve with 7 points, ranging from 0.156 - 10ng/mL.

a) Centrifuge the **HspA6 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: 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL, 0.3125ng/mL, 0.156ng/mL.

c) Add 500µL of **Sample Diluent** to Tube #1.
d) Add 250µL of **Sample Diluent** to Tube #2, 3, 4, 5, 6 and 7.
e) Add 5µL of the **HspA6 Standard** stock solution (1µg/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 #7.
j) Finally, add 250µL **Sample Diluent** to another 1.5mL polypropylene tube (Tube # 8), which is the zero standard (0ng/mL).



3. WASH BUFFER

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 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-HSPA6

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

b) Dilute 22µL of **Anti-HspA6** in 11mL of **Anti-HspA6 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 **Anti-HspA6**.

5. HRP CONJUGATE

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

b) Dilute 22µL of **HRP Conjugate** in 11mL 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 Hsp70B' Plate Template on page 23 to determine the number of wells to be used.
   b) Remove the **Anti-HspA6 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 **HspA6 Standard** (Tube#1 through Tube #7)
      - Samples (previously prepared - see Sample Preparation, page 9)
      - 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 2 hours.

***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-HSPA6
(*previously diluted, see page 12)*

a) Add 100µL of the previously diluted **Anti-HspA6** 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 12)*

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 1 hour.
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 2 minutes 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 450 nm.
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 HSP70B' 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).

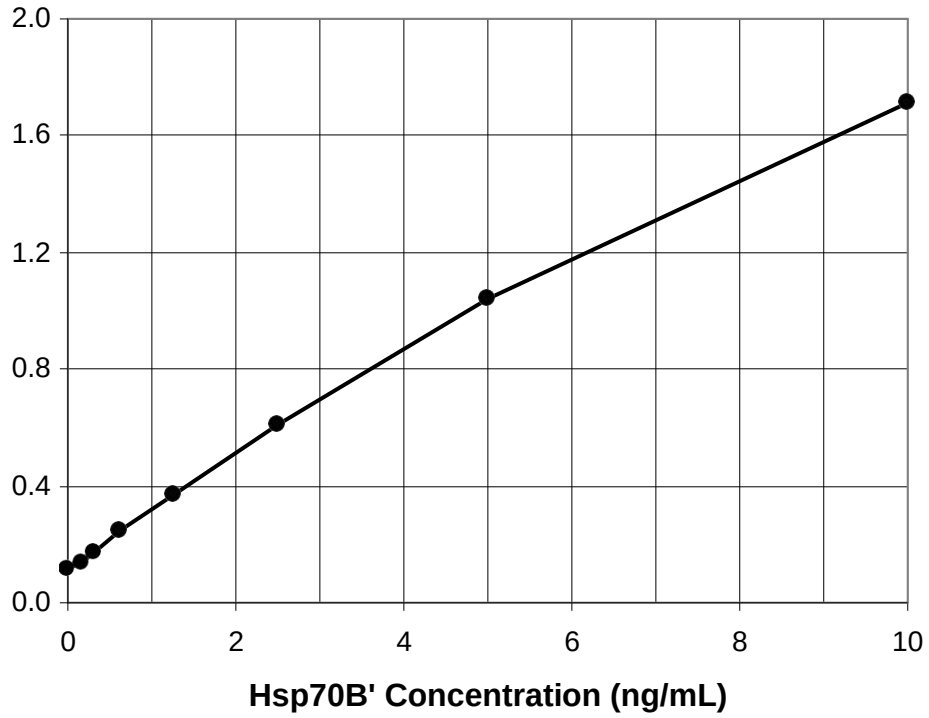
4. To generate the standard curve, calculate the log of each standard concentration and the corresponding mean net optical density produced in the assay. On a linear to linear scale, plot the log (concentrations) on the X-axis, and the log (absorbance measurements) on the Y-axis. Determine the best fit line.

5. Interpolate the sample concentrations from the standard curve and multiply by the dilution factor for the final sample HspA6 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 HspA6 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 HSPA6 STANDARD CURVE



PERFORMANCE CHARACTERISTICS

1. SENSITIVITY

   The sensitivity of the HspA6 EIA has been determined to be 0.062 ng/mL. The standard curve has a range of 0.156 to 10 ng/mL.

2. PRECISION

   a) Intra-Assay Precision (Within Run Precision)
   To determine Intra-Assay Precision, three samples of known concentration were assayed twenty times on one plate.
   The Intra-Assay Coefficient of variation of the HspA6 EIA 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 multiple times in several individual assays. The Inter-Assay Coefficient of variation of the HspA6 EIA has been determined to be <10%.

3. SPECIFICITY AND SPECIES REACTIVITY

The HspA6 EIA detects human Hsp70B' and does not cross react with Hsp70, Hsc70, Hsp71 *(M. tuberculosis)*, Grp78, DnaK (*E. coli*).

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 HspA6 Extraction Reagent that will be required. For every 1 X 10^6 to 1 X 10^7 cells, use 1mL of 1X HspA6 Extraction Reagent.
6. Dilute an appropriate amount of 5X HspA6 Extraction Reagent with cold (4°C) deionized or distilled water to generate the required volume of 1X HspA6 Extraction Reagent. For example, if 5mL of 1X HspA6 Extraction Reagent were required, dilute 1mL of the 5X HspA6 Extraction Reagent with 4mL of cold deionized or distilled water.
7. Add protease inhibitors to the 1X HspA6 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 HspA6 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 HspA6 EIA 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.

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 HspA6 Extraction Reagent that will be required. For each ~0.5cm^3 piece of tissue, use 1mL of 1X HspA6 Extraction Reagent.
4. Dilute an appropriate amount of 5X HspA6 Extraction Reagent with cold (4°C) deionized or distilled water to generate the required volume of 1X HspA6 Extraction Reagent. For example, if 5mL of 1X HspA6 Extraction Reagent were required, dilute 1mL of the 5X HspA6 Extraction Reagent with 4mL of cold deionized or distilled water.
5. Add protease inhibitors to the 1X HspA6 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 HspA6 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 HspA6 EIA 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 HspA6 EIA 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 HspA6 EIA kit. Although the effect of every chemical is not known, Novus Biologicals 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 Hsp70B' in a sample is inhibited by ≤10%.

CHEMICAL	COMPATIBLE LIMIT
Aprotinin	50µg/mL
ß-mercaptoethanol	0.75mM
CHAPS	0.5% (w/v)
Dithiothreitol (DTT)	1mM
EDTA	100mM
Glycerol	1% (v/v)
HEPES, pH 7.5	25mM
Leupeptin	50µg/mL
Magnesium Chloride ($MgCl_2$)	500mM
MOPS, pH 7.5	250mM
NP-40	1% (v/v)
Pepstatin A	50µg/mL
PMSF	50mM
SDS	0.01% (w/v)
Sodium Azide (NaN3)	2.5% (w/v)
Sodium Deoxycholate	0.1% (w/v)
Sodium Chloride (NaCl)	500mM
Sodium Phosphate, pH 7.2	150mM
Tris, pH 7.5	250mM
Triton-X100	1% (v/v)
Tween-20	1% (v/v)

APPENDIX V - HspA6 Immunoassay Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	1.25ng/mL	0ng/mL									
B	Blank	1.25ng/mL	0ng/mL									
C	10ng/mL	0.625ng/mL										
D	10ng/mL	0.625ng/mL										
E	5ng/mL	0.3125ng/mL										
F	5ng/mL	0.3125ng/mL										
G	2.5ng/mL	0.156ng/mL										
H	2.5ng/mL	0.156ng/mL										

APPENDIX VI - Nomenclature of Hsp70 Family Members

Hsp70 family member	**Form**
Hsc70 or Hsp73	Constitutive-cytosolic / nuclear
Hsp70 or Hsp72, Hsp70-1, Hsp70-2, Hsp70i, Hsx70	Inducible-cytosolic / nuclear
BiP or Grp78	Endoplasmic reticulum (ER)
mito-Hsp70 or Grp75	Mitochondrial
SsaI-4	Yeast homolog of Hsc70
Kar2	Yeast homolog of BiP
DnaK	E.coli Hsp70
Hsp70B	5’ gene fragment (heat-inducible promoter) - no functional gene product (8,9)
HspA6 or HspA6 or Hsp70-6	Heat-inducible form

REFERENCE

1. Bring to room temperature: **Anti-HspA6 Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent**, **Anti-HspA6 Diluent**, **HRP Conjugate Diluent, TMB Substrate** and **Stop Solution 2**.
2. Prepare **Recombinant HspA6 Standard** and samples in **Sample Diluent**.
3. Add 100µL prepared standards and samples in duplicate to wells of **Anti-HspA6 Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 2 hours.
5. Wash wells 6X with 1X Wash Buffer.
6. Add 100µL diluted **Anti-HspA6** 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 1 hour.
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 HspA6 standard curve and calculate Hsp70B' 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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