Product Manual

HSP70 ELISA kit

Catalog #: ADI-EKS-700B

For the detection and quantitation of Hsp70 in cell lysates and tissue extracts.

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Product Manual

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Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

INTRODUCTION

The traditional method for Hsp70 detection and quantitation is accomplished in two steps: immunoblotting followed by densitometry scanning. The Enzo Life Sciences Hsp70 ELISA kit provides researchers with a rapid and reliable method to measure the levels of inducible Hsp70 in numerous samples.

This kit has the potential of expanding our knowledge of Hsp70's role in critical cellular processes or implicating Hsp70 as a diagnostic tool to evaluate and monitor a variety of diseases.

SCIENTIFIC OVERVIEW

Inducible heat shock protein 70 (Hsp70) is a stress protein whose expression is upregulated when the cell or organism is placed under conditions of stress. Hsp70 is essential for cellular recovery, survival, and maintenance of normal cellular function.[1] Hsp70 is also a molecular chaperone that prevents protein aggregation and refolds damaged proteins in response to cellular stress caused by environmental insults, pathogens, and disease. Current research is aimed at exploiting Hsp70's cellular protective abilities as a therapeutic strategy against damaging cellular stress.[26,27]

In most mammals, the expression of inducible Hsp70 is strictly stress inducible and can only be detected following a significant stress upon the cell or organism.[2, 3] However, in humans and primates, inducible Hsp70 is present at basal levels and is upregulated in response to stress.[4, 5, 6] The role of Hsp70 has been studied in a variety of medically relevant models or conditions such as hyperthermia,[7] hypertension,[8] toxic exposure to chemical agents,[9] hypoxia,[10] ischemia,[11, 12] inflammation,[13] autoimmunity,[8, 14] apoptosis,[15, 16] cancer,[16] organ transplantation,[17] and bacterial[18, 19] and viral[20] infections. Hsp70 has also been studied in the normal processes of aging,[15, 21] spermatogenesis,[22, 23] menstruation,[24] and physical activity such as exercise.[25]

ASSAY DESIGN

The Enzo Life Science Hsp70 ELISA (enzyme-linked immunosorbent assay) provides a method to detect and quantitate inducible Hsp70 in samples from human, mouse, and rat origins. This assay allows for the reproducible, accurate, and precise determination of inducible Hsp70 from cell lysates and tissue extracts. **(Please note that this assay is not appropriate for use with serum or plasma samples.)** The assay is specific for both native and recombinant inducible Hsp70 and it does not detect other Hsp70 family members, such as Hsc70, Grp78, DnaK (E. coli), recombinant M. tuberculosis Hsp71 or human Hsp60.

The Hsp70 ELISA is a quantitative sandwich immunoassay. A mouse monoclonal antibody specific for inducible Hsp70 is pre-coated on the wells of the provided Hsp70 Immunoassay Plate. Inducible Hsp70 is captured by the immobilized antibody and is detected with a Hsp70 specific rabbit polyclonal antibody. The rabbit polyclonal antibody is subsequently bound by a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody. The assay is developed with tetramethylbenzidine (TMB) substrate and a blue color develops in proportion to the amount of captured Hsp70. 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. Hsp70 concentrations from the sample are quantitated by interpolating absorbance readings from a standard curve generated with the calibrated Hsp70 protein standard provided.

ASSAY PROCEDURE SUMMARY

1. Bring the appropriate reagents to room temperature.
2. Prepare **Recombinant Hsp70 Standard** and samples in **Sample diluent 2.**
3. Add 100µl prepared standards and samples in duplicate to wells of **Anti-Hsp70 Immunoassay Plate.** Cover immunoassay plate.
4. Incubate plate at room temperature for 2 hours.
5. Wash wells 4X with 1X Wash Buffer.
6. Add 100µl **Hsp70 Antibody** to each well. Cover immunoassay plate.
7. Incubate plate at room temperature for 1 hour.
8. Wash wells 4X with 1X Wash Buffer.
9. Add 100µl **Hsp70 Conjugate** to each well. Cover immunoassay plate.
10. Incubate plate at room temperature for 1 hour.
11. Wash wells 4X with 1X Wash Buffer.
12. Add 100µl **TMB Substrate** to each well.
13. Incubate at room temperature for 30 minutes.
14. Add 100µl **Stop Solution 2** to each well.
15. Measure absorbance at 450nm, or 450nm with a correction at 540 or 570nm.
16. Plot the Hsp70 standard curve and calculate Hsp70 sample concentrations.

MATERIALS PROVIDED

The Enzo Life Sciences Hsp70 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
80-1581	Anti-Hsp70 Immunoassay Plate	96 well plate	12 x 8 removable strips and frame. Pre-coated plate with mouse monoclonal antibody specific for inducible Hsp70.
80-1526	5X Extraction Reagent	10ml	Concentrated buffer for preparation of cell and tissue extracts.
80-1776	Recombinant Hsp70 Standard	25µl	10µg/ml stock solution of inducible Hsp70 protein.
80-1617	Sample Diluent 2	50ml	Buffer to dilute standards and samples.
80-1287	20X Wash Buffer	100ml	Concentrated solution of buffer and surfactant.
80-1593	Hsp70 Antibody	10ml	Rabbit polyclonal antibody specific for inducible Hsp70.
80-1594	Hsp70 Conjugate	10ml	Horseradish peroxidase conjugated anti-rabbit IgG.
80-0350	TMB Substrate	10ml	Stabilized tetramethylbenzidine substrate.
80-0377	Stop Solution 2	10ml	Acid solution to stop color reaction.

STORAGE

All reagents are stable as supplied at 4°C, except the **Recombinant Hsp70 Standard** which should be stored at -20°C. If assaying on two or more separate occasions, aliquot the standard into smaller portions and store at -20°C. Unused wells of the **Anti-Hsp70 Immunoassay Plate** should be resealed, along with accompanying desiccant pack, in the foil pouch provided and stored at 4°C until the kit's expiry date.

OTHER MATERIALS NEEDED

- Deionized or distilled water
- Precision pipettors capable of accurately delivering 5 to 1,000µl
- Disposable pipette tips
- 5, 10, 25ml pipettes for reagent preparation
- 2L 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

CRITICAL ASSAY PARAMETERS AND NOTES

- The Enzo Life Sciences Hsp70 ELISA kit contains a precoated microtiter plate **(Anti-Hsp70 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.
- **Please note that this assay is *not* appropriate for use with serum or plasma samples.**
- Run both standards and samples in duplicate.
- Include a standard curve each time the assay is performed.
- Standards should be freshly prepared prior to use. Standards and samples should be prepared **on ice** and used within 30 minutes of preparation.

- The following kit components should be brought to room temperature prior to use: **Anti-Hsp70 Immunoassay Plate, 20X Wash Buffer, Sample Diluent 2, Hsp70 Antibody, Hsp70 Conjugate, 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 20 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.

  NOTE: *This assay has been validated for use with cell lysates and tissue extracts.*

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

SAMPLE PREPARATION

1. EXTRACTION OF SAMPLES

Suggested protocols for the preparation of cell lysates and tissue extracts, may be found in Appendices I-II, respectively (see pages 20-23). **Please note that this assay is *not* appropriate for use with serum or plasma samples**. 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 III (see page 24).

2. DILUTION OF SAMPLES

Samples should be prepared as described in Appendices I-II. Sample Diluent 2 should be used to dilute lysates and tissue extracts and accompanying standards. Please note that this assay is *not* appropriate for use with serum or plasma samples.

A minimum 1:4 dilution of 1X Extraction Reagent into Sample Diluent 2 is required to remove matrix interference of this buffer. Due to differences in sample types, number of cells, or total cellular protein concentration, samples may require greater dilution with Sample Diluent 2 to remove interference or to be read within the range of the standard curve. Users must determine the optimal sample dilutions for their particular experiments. Below are examples of sample recoveries with several different types of samples. Note that % recovery was calculated based on linearity of samples.

Sample	Total cellular protein (mg/ml)	Recovery (%)	Recommended Dilution
HeLa cells	1.336	109	1:80
heat-shocked HeLa cells	1.296	106	1:80
human liver microsomes	2.0	97	1:4

a) Dilute prepared samples (i.e. cell and tissue lysates) in **Sample Diluent 2**. 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 4). Samples must be kept **on ice** while reagents are being prepared.

REAGENT PREPARATION

NOTE: *Standards should be freshly prepared prior to use. Standards and samples should be prepared* ***on ice*** *and used within 30 minutes of preparation.*

1. TEMPERATURE OF REAGENTS

Bring the following reagents to room temperature prior to use:

- Anti-Hsp70 Immunoassay Plate *(part#: ADI-80-1581)*
- Sample Diluent 2 *(part#: ADI-80-1617)*
- Wash Buffer *(part#: ADI-80-1287)*
- Hsp70 Antibody *(part#:ADI-80-1593)*
- Hsp70 Conjugate *(part#:ADI-80-1594)*
- TMB Substrate *(part#: ADI-80-0350)*
- Stop Solution 2 *(part#: ADI-80-0377)*

2. HSP70 STANDARD CURVE (part#: 80-1776)

The Recombinant **Hsp70 Standard** is used to generate a standard curve with 7 points, ranging from 0.78 - 50ng/ml.

a. Centrifuge the **Hsp70 Standard** vial before removing cap.

b. Label seven (7) polypropylene tubes, each with one of the following standard values: 50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, 3.125 ng/ml, 1.56 ng/ml, 0.78 ng/ml.

c. Add 995µl of **Sample Diluent 2** to Tube #1 (50ng/ml).

d. Add 500µl of **Sample Diluent 2** to Tubes #2, 3, 4, 5, 6, and 7.

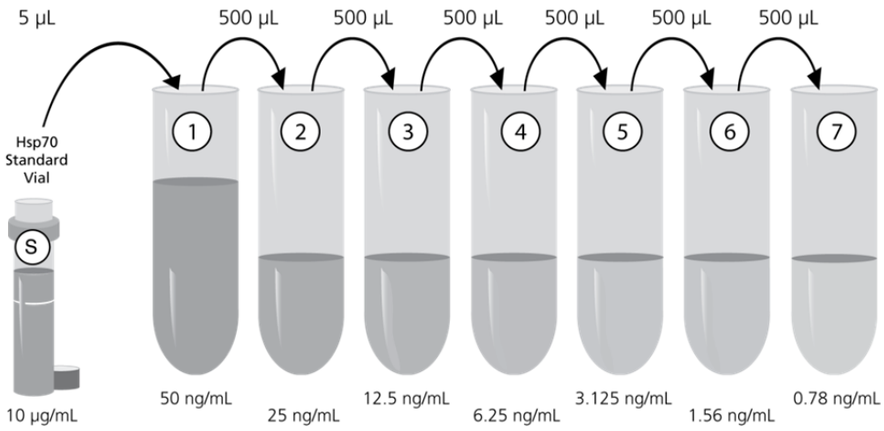
e. Add 5µl of the **Hsp70 Standard** stock solution (10µg/ml) to Tube #1 (50ng/ml).

f. Mix thoroughly.

g. Transfer 500µl from Tube#1 (50ng/ml) to Tube #2 (25ng/ml).

h. Mix thoroughly.

i. Similarly, complete the dilution series to generate the remaining standards (500µl from Tube #2 to Tube #3, mix thoroughly, etc.), up to and including Tube #7.



j. Finally, add 500µl Sample Diluent 2 to another polypropylene tube (Tube #8), which is the assay blank (0 ng/ml).

NOTE: *The* ***Recombinant Hsp70 Standard*** *will withstand a freeze/thaw cycle to allow preparation of a second standard curve. However, to ensure product integrity, any remaining Hsp70 Standard should be discarded after the second use.*

3. WASH BUFFER (part#: 80-1287)

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

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

NOTE: *100ml of 20X Wash Buffer has been provided in this kit, which is sufficient for the preparation of 2L of 1X Wash Buffer. The minimum required volume of 1X Wash Buffer is 460 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.*

ASSAY PROCEDURE

1. DETERMINE THE REQUIRED NUMBER OF WELLS

a. If less than 96 pre-coated microtiter wells are needed, remove the excess wells from the frame and return them, along with the accompanying desiccant, to the foil pouch.

b. Reseal the pouch containing the unused wells and desiccant 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 **Hsp70 Standard Curve** (Tube#1 through Tube #7)
- Samples (previously prepared - see Sample Preparation, pages 8-9)
- **0 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 20 minutes.

3. WASHING

a. Aspirate liquid from all wells.

b. Add 400µl 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 3 more times, for a total of 4 washes.

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

4. ADDITION OF HSP70 ANTIBODY

a. Add 100µl of the Hsp70 Antibody into 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 HSP70 CONJUGATE**
   a. Add 100µl of the **Hsp70 Conjugate** into 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 into each well. Color development should be visible within 1 minute of addition to the plate.
   b. Incubate the plate at room temperature for 30 minutes.
   c. Add 100µl of the **Stop Solution 2** into each 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. If the reader is capable of measuring at dual wavelengths, set the correction wavelength at 540 or 570nm.
   c. Measure the absorbance. If the plate cannot be read immediately, it should be covered and kept at room temperature. The absorbance should be read within 30 minutes of adding the Stop Solution.

CALCULATION OF RESULTS -

DETERMINATION OF HSP70 CONCENTRATIONS

Several options are available for the calculation of the concentration of Hsp70 in samples. We recommend that the data be handled by an immunoassay software package utilizing a 4-parameter logistic curve fitting program. If data reduction software is not readily available, the concentrations can be calculated as follows:

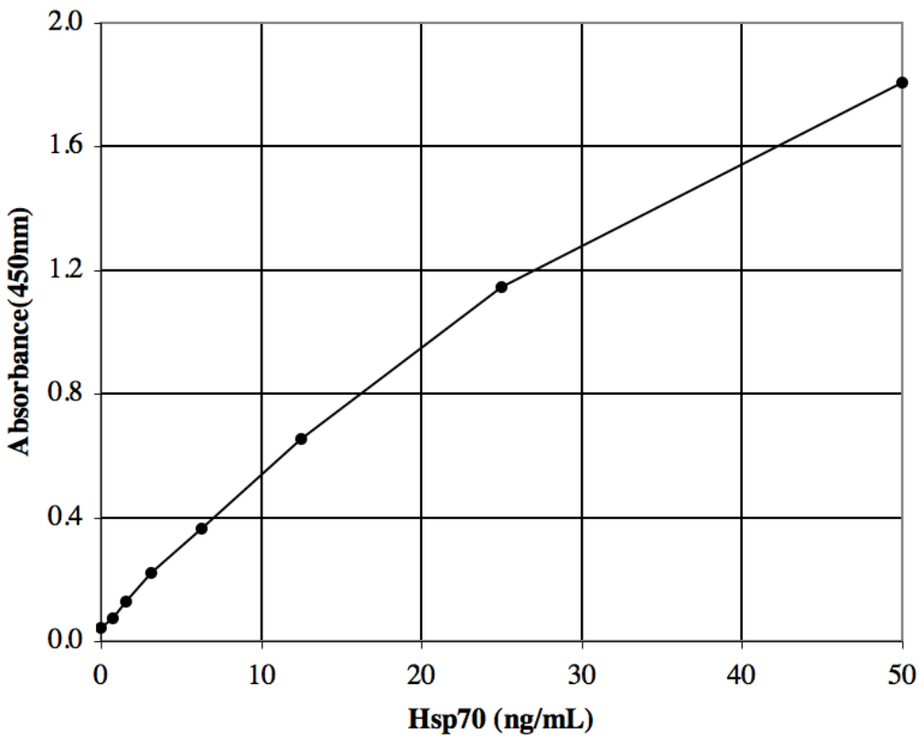
1. Calculate the average Net OD for each standard and sample by subtracting the average blank OD from the average OD for each standard and sample.

   Average Net OD = Average OD – Average Blank OD

2. Plot the average Net OD for each standard versus Hsp70 concentration in each standard. Approximate a straight line through the points.

3. Interpolate the sample concentrations from the standard curve and multiply by the dilution factors for the final sample Hsp70 concentrations. 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 Hsp70 concentration.

TYPICAL HSP70 STANDARD CURVE



PERFORMANCE CHARACTERISTICS

1. SENSITIVITY

The sensitivity was calculated by first multiplying the concentration of the Low Standard (0.78 ng/ml) by two (2) standard deviations of the mean OD of twenty-four (24) replicates of 0 ng/ml Standard. This value was then divided by the difference between the mean OD of twenty-four (24) replicates of the Low Standard and the mean OD of the twenty-four (24) replicates of 0 ng/ml Standard.

The sensitivity of the Hsp70 ELISA has been determined to be 0.2 ng/ml.

The standard curve has a range of 0.78-50 ng/ml.

2. PRECISION

a. **Intra-Assay Precision** (Within Run Precision)

To determine Intra-Assay Precision, 3 samples of known concentration were assayed 24 times on one plate. The Intra-Assay Coefficient of variation of The Enzo Life Sciences Hsp70 ELISA has been determined to be < 5%.

b. **Inter-Assay Precision** (Between Run Precision)

To determine Inter-Assay Precision, 3 samples of known concentration were assayed 9 times in individual assays. The Inter- Assay Coefficient of variation of The Enzo Life Sciences Hsp70 ELISA has been determined to be < 13%.

3. LINEARITY

A buffer sample containing 30 ng/ml of recombinant Hsp70 was serially diluted 1:2 in **Sample Diluent 2** and measured in the assay. The results are shown in the table below.

Sample Diluent 2

Dilution	Expected (ng/ml)	Observed (pg/ml)	Recovery (%)
Neat		30.669	
1:2	15.335	15.165	98.89
1:4	7.667	7.94	103.56
1:8	3.834	4.037	105.31
1:16	1.917	1.989	103.77
1:32	0.958	0.935	97.56

4. RECOVERY

Recovery was determined by running serial dilutions of relevant samples (cell lysate and tissue extract) in **Sample Diluent 2**. The observed concentration of each sample was interpolated from the standard curve and then multiplied by the dilution performed to give the final sample concentration. Linearity was calculated at each dilution (excluding the last dilution).

Recoveries in which linearity fell between 85% and 115% were averaged to calculate the % recovery. The % recoveries for cell lysates and tissue extracts were $\geq$ 90%.

The percent linearity was calculated by dividing the final concentration of each dilution by the final concentration of the last dilution, whose observed concentration fell within the standard range. Linearity of the samples was achieved at or above 85%.

5. SPECIFICITY AND SPECIES REACTIVITY

The Enzo Life Sciences Hsp70 ELISA is specific for both natural source and recombinant inducible Hsp70. The assay does not cross react with 5,000ng/ml of bovine constitutive Hsc70, recombinant hamster Grp78, *E. coli* DnaK, or recombinant *M. tuberculosis* Hsp71. The Hsp70 ELISA has been certified for the detection of human, mouse, and rat inducible Hsp70.

LIMITATIONS OF THE ASSAY

- This assay has been validated for use with cell lysates and tissue extracts. 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. Please note that this assay is *not* appropriate for use with serum or plasma samples.
- Although this assay has been validated for use with cell lysates and tissue extracts, 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.

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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 106 to 1 X 107 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, making sure 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. 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 labelled 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 Hsp70 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 and stored at -70°C. The extract can be 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 labelled polypropylene tube. The supernatant collected is the tissue extract, which is now ready for analysis using the Hsp70 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

CHEMICAL COMPATIBILITY LIMITS

Different chemicals may interfere with the Hsp70 ELISA Kit. Although the effect of every chemical is not known, Stressgen has tested the following chemicals to determine the levels at which they may interfere with the kit. The compatible limit is defined as the chemical concentration at which the measurement of Hsp70 in a sample is inhibited by ≤10%.

Chemical	Compatible Limit
Aprotinin	10µg/ml
β-mercaptoethanol	<0.15mM
CHAPS	0.1% (w/v)
Dithiothreitol (DTT)	<0.2mM
EDTA	20mM
GLYCEROL	<0.2% (v/v)
HEPES, pH 10	5mM
Leupeptin	10µg/ml
Magnesium Chloride ($MgCl_2$)	100mM
MOPS, pH 7.5	50mM
NP-40	1%
Pepstain	10µg/ml
PMSF	1mM
RIPA Buffer	5%
SDS	0.01%
Sodium Azide (NaN_3)	0.5% (w/v)
Sodium Chloride (NaCl)	150mM
Sodium Phosphate, pH 7.2	<125mM
Tris, pH 7.5	<50mM
Triton-X 100	1%
Tween-20	1%

APPENDIX IV

HSP70 IMMUNOASSAY PLATE TEMPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	0ng/ml	0ng/ml									
B	Blank	6.25ng/ml	0ng/ml									
C	50ng/ml	3.125ng/ml										
D	50ng/ml	3.125ng/ml										
E	25ng/ml	1.56ng/ml										
F	25ng/ml	1.56ng/ml										
G	12.5ng/ml	0.78ng/ml										
H	12.5ng/ml	0.78ng/ml										

REFERENCE

1. Bring to room temperature: **Anti-Hsp70 Immunoassay Plate**, **20X Wash Buffer**, **Sample Diluent 2**, **Hsp70 Antibody, Hsp70 Conjugate, TMB Substrate,** and **Stop Solution 2**.
2. Prepare **Recombinant Hsp70 Standard** and samples in **Sample Diluent 2**. Please note that this assay is *not* appropriate for use with serum or plasma samples.
3. Add 100µl prepared standards and samples in duplicate to wells of **Anti-Hsp70 Immunoassay Plate**. Cover immunoassay plate.
4. Incubate plate at room temperature for 2 hours.
5. Wash wells 4X with 1X Wash Buffer.
6. Add 100µl **Hsp70 Antibody** to each well. Cover immunoassay plate.
7. Incubate plate at room temperature for 1 hour.
8. Wash wells 4X with 1X Wash Buffer.
9. Add 100µl **Hsp70 Conjugate** to each well. Cover immunoassay plate.
10. Incubate at room temperature for 1 hour.
11. Wash wells 4X with 1X Wash Buffer.
12. Add 100µl **TMB Substrate** to each well.
13. Incubate at room temperature for 30 minutes
14. Add 100µl **Stop Solution 2** to each well.
15. Measure absorbance at 450nm, with a correction at 540 or 570nm.
16. Plot the Hsp70 standard curve and calculate the Hsp70 sample concentrations.

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