HSP 70 ELISA Kit

Enzyme Immunoassay for the quantitative determination of HSP70 in Human, Dog and Goat cell lysates and tissue lysates.

Catalog number: ARG81393

Package: 96 wells

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INTRODUCTION

HSP70 is a 70kDa heat shock protein which is a member of the heat shock protein 70 family. In conjuction with other heat shock proteins, this protein stabilizes existing proteins against aggregation and mediates the folding of newly translated proteins in the cytosol and in organelles. It is also involved in the ubiquitin-proteasome pathway through interaction with the AU-rich element RNA-binding protein 1. The gene is located in the major histocompatibility complex class III region, in a cluster with two closely related genes which encode similar proteins. [provided by RefSeq, Jul 2008]

HSP70 is in cooperation with other chaperones, Hsp70s stabilize preexistent proteins against aggregation and mediate the folding of newly translated polypeptides in the cytosol as well as within organelles. These chaperones participate in all these processes through their ability to recognize nonnative conformations of other proteins. They bind extended peptide segments with a net hydrophobic character exposed by polypeptides during translation and membrane translocation, or following stress-induced damage. In case of rotavirus A infection, serves as a post-attachment receptor for the virus to facilitate entry into the cell. Essential for STUB1-mediated ubiquitination and degradation of FOXP3 in regulatory T-cells (Treg) during inflammation. [UniProt]

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. This kit is designed to react human HSP70, however, this kit is also used to detect dog and goat HSP70 protein from dog and goat samples.

An antibody specific for HSP70 has been pre-coated onto a microtiter plate. Standards or samples are pipetted into the wells and any HSP70 present is bound by the immobilized antibody. After washing away any unbound substances, a biotin-conjugated antibody specific for HSP70 is added to each well and incubate. Following a washing to remove unbound substances, streptavidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After washing away any unbound antibody-enzyme reagent, a substrate solution (TMB) is added to the wells and color develops in proportion to the amount of HSP70 bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450nm ±2nm. The concentration of HSP70 in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at -4°C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	96 wells	4°C
Standard (Lyophilized)	2 vials	4°C
Standard/Sample diluent	50 ml (ready to use)	4°C
100X Antibody conjugate concentrate	150 µl	4°C
Antibody diluent buffer	13 ml (ready to use)	4°C
100X HRP-Streptavidin concentrate	150 µl	4°C
HRP-Streptavidin diluent buffer	13 ml (ready to use)	4°C
5X Extraction Reagent	10 ml	4°C
Pre-treatment Buffer	13 ml (ready to use)	4°C
10X Wash buffer	100 ml	4°C
TMB substrate	13 ml (ready to use)	4°C (Protect from light
STOP solution	13 ml (ready to use)	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes and pipette tips
- Ultra-pure water, deionized or distilled water
- Polypropylene or polyethylene tubes to prepare samples – do not use polystyrene, polycarbonate or glass tubes.
- Protease inhibitors
- Adhesive plate sealers

- Microplate shaker (shaking amplitude 3 mm; approx. 600 rpm) or orbital shaker with suitable speed
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times. Unused wells should be resealed with desiccant in the foil pouch provided, and stored at 4°C until the kits expiry date.
- If crystals are observed in the 10X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- All materials should be equilibrated to room temperature (RT, 22-25°C) 20 min before use.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- Mix the contents of the microplate wells thoroughly by microplate shaker for few seconds or gently tap the plate to ensure good test results. Please mix carefully to avoid well-to-well contamination. Do not reuse microwells.
- The TMB substrate should be colorless and transparent before using. Take care not to contaminate the TMB Substrate. Do not expose TMB

Substrate solution to glass, foil, or metal. Do NOT cover the plate with aluminum foil or metalized mylar film. If the solution is blue before use, DO NOT USE IT.

- Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur.
- Use a new adhesive plate cover for each incubation step.
- Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
- Avoid using reagents from different batches.
- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Lysate Preparation-

1. Prepare and treat cells as desired.
2. a. For adherent cells- Remove media and rinse cells with ice-cold PBS. Harvest cells with trypsin-EDTA or by using a cell scraper. Centrifuge at 500 x g for 5 minutes.

   b. For suspension cells- Harvest by centrifugation at 500 x g for 5 minutes.
3. Wash cells by re-suspending the cell pellet in ice-cold PBS. Pellet cells by centrifugation at 500 x g for 5 minutes. Repeat wash for a total of three washes with ice-cold PBS.
4. Use a pipette to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible. The cell pellet may be frozen at -70°C and lysed at a later time, if desired.
5. Calculate the amount of 1X Extraction Reagent required. For every 1 X 10^6 to 1X 10^7 cells, use 1 mL of 1X Extraction Reagent.

   Note: Use of alternative extraction buffers may contain components which could interfere and compromise the performance of the assay, producing inaccurate results. For best results, use the 1X Extraction Reagent included in this kit.
6. Add protease inhibitors to the 1X Extraction Reagent before use. Examples of an appropriate protease inhibitor cocktail includes 0.1 mM

PMSF, 1 µg/mL leupeptin, 1 µg/ mL aprotinin, and 1 µg/mL pepstatin. Alternatively, a commercially available protease cocktail, obtainable from a variety of scientific reagent vendors, may also be used.

7. Add appropriate amount of ice-cold 1X Extraction Reagent including protease inhibitors to the cell pellet.
   Note: If excess buffer is used for the number of cells lysed, the protein concentration will be low.
8. Pipet up and down to break up the cell pellet until the cell suspension is homogeneous and no clumps are visible.
9. Incubate on ice for 30 minutes with occasional mixing or sonication.
   Note: To increase protein yields and decrease sample viscosity, aspirate the cell pellet 5-10 times through a 21 ½ gauge needle or sonicate the cell pellet for 30 seconds with 50% pulse during the incubation.
10. Transfer the mixture to a fresh micro centrifuge tube and centrifuge at ~21,000 x g for 10 minutes at 4°C.
11. Transfer the supernatant (cell lysate) to a fresh tube for analysis. Avoid disturbing the cell pellet. Discard the cell pellet once the supernatant is harvested. The cell lysate is now ready for analysis in the assay.
12. Alternatively, store the cell lysate in single-use aliquots at -70°C. It is recommended that a protein determination assay be performed and the extracts aliquoted into convenient amounts prior to storing at -70°C to avoid multiple freeze-thaw cycles.

Tissue Extract Preparation-

1. Harvest tissue to be analyzed. Tissues may be flash frozen, stored at -70°C and prepared at a later time, if desired.
2. Calculate the amount of Extraction Reagent required. For each 0.5 cm^3 piece of tissue, use 1 mL of 1X Extraction Reagent.

   Note: For best results, use the 1X Extraction Reagent included in this kit. Use of alternative extraction buffers may contain interfering components and compromise assay performance.
3. Add protease inhibitors to the 1X Extraction Reagent. Examples of an appropriate protease inhibitor cocktail includes 0.1 mM PMSF, 1 µg/mL leupeptin, 1 µg/ mL aprotinin, and 1 µg/mL pepstatin. Alternatively, a commercially available protease cocktail, obtainable from a variety of scientific reagent vendors, may also be used.
4. Place the tissue in a mortar and add sufficient volume of liquid nitrogen to cover the tissue.
5. Allow the liquid nitrogen to evaporate. The tissue should be thoroughly frozen.
6. Grind the frozen tissue to a powder with a pestle.
7. Add appropriate amount of ice-cold 1X Extraction Reagent including protease inhibitors to the processed tissue. Continue to homogenize the tissue with the pestle until the tissue suspension is homogeneous.
9. Transfer the extract to a fresh micro centrifuge tube and centrifuge at ~21,000 x g for 10 minutes at 4°C.
10. Transfer the supernatant (tissue extract) to a fresh tube for analysis. Avoid disturbing the cell pellet. Discard the cell pellet once the

supernatant is harvested. The tissue extract is now ready for analysis in the assay.

11. Alternatively, store the tissue extract in single-use aliquots at -70°C. It is recommended that a protein determination assay be performed and the extracts aliquoted into convenient amounts prior to storing at -70°C to avoid multiple freeze-thaw cycles.

Note:

a) Do not use haemolytic, icteric or lipaemic specimens.

b) Samples containing sodium azide should not be used in the assay.

c) If particulate is present in samples, centrifuge prior to analysis.

REAGENT PREPARATION

- 1X Extraction Reagent: Prepare freshly before use. Dilute 5X Extraction Reagent into ice-cold distilled water to yield 1X Extraction Reagent. (E.g. 1 ml of 5X Extraction Reagent + 4 ml of ice-cold distilled water). It is recommended preparing only the required amount of 1X Extraction Reagent,
- 1X Wash buffer: Dilute 10X Wash buffer into distilled water to yield 1X Wash buffer. (E.g. 100 ml of 10X Wash buffer + 900 ml of distilled water) The diluted Wash buffer is stable for one month at 4°C. Do not use 1X Wash Buffer if it becomes visibly contaminated during storage.
- Antibody conjugate working solution: Prior to use, prepare only the required amount of 1X Antibody conjugate working solution for the number of strips being used. Dilute Antibody conjugate concentrate (100X) into Antibody diluent buffer and mix well to yield 1X Antibody conjugate working solution. (E.g. 6 ml of 1X Antibody conjugate working

solution is required for 48 wells used: 60 µl of Antibody conjugate concentrate (100X) + 6 ml of Antibody diluent buffer, mix well)

- **HRP-Streptavidin working solution**: Prior to use, prepare only the required amount of 1X HRP-Streptavidin working solution for the number of strips being used. Dilute HRP-Streptavidin concentrate (100X) into HRP-Streptavidin diluent buffer and mix well to yield 1X HRP-Streptavidin conjugate working solution. (E.g. 6 ml of 1X HRP-Streptavidin working solution is required for 48 wells used: 60 µl of HRP-Streptavidin conjugate concentrate (100X) + 6 ml of HRP-Streptavidin diluent buffer, mix well)
- **Sample Dilution:** Samples must first be diluted prior to testing.
  - ➢ Suggested starting dilutions for samples:
  - For cell and tissue lysates, dilute samples at 4X dilution with Standard/Sample diluent. For example, dilute 60 µL of sample in 180 µl of Standard/Sample diluent. Mix well.

  Note: If values for samples are not within the range of the standard curve, optimal sample dilutions need to be determined.
  - ➢ Prepare at least 120 µL of sample in Standard/Sample diluent for duplicates. Mix samples well prior to analysis.
- **Standards:** Reconstitute the standard with **1 ml** of Standard/Sample diluent to yield a stock concentration of **66.7 ng/ml**. Keep the buffer in the vail for at least 5 min at RT to make sure the standard is dissolved completely before making serial dilutions. The Standard/Sample diluent buffer serves as zero standard (0 ng/ml), and the rest of the standard serial dilution can be diluted as according to the suggested

concentration below: 50 ng/ml, 25 ng/ml, 12.5ng/ml, 6.25 ng/ml, 3.125 ng/ml, 1.563 ng/ml, 0.781 ng/ml.

Dilute HSP70 standard as according to the table below:

Standard	HSP70 Conc. (ng/ml)	µl of Diluent	µl of standard
S7	50	250	750 (66.7 ng/ml stock)
S6	25	250	250 (S7)
S5	12.5	250	250 (S6)
S4	6.25	250	250 (S5)
S3	3.125	250	250 (S4)
S2	1.563	250	250 (S3)
S1	0.781	250	250 (S2)
S0	0	250	0

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-25°C.) before use. Standards, samples and controls should be assayed in duplicates.

1. Remove excess microtiter strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it. Store unused strips at 2-8°C.
2. Add 100 µl of the Standards into the appropriate wells.
3. Add 50 µl of Pre-Treatment Buffer to all sample wells.
4. And then add 50 µl of appropriately diluted sample to each sample well.
5. Gently tap the plate to mix well. Cover the plate and incubate for 1 h at 37°C on a microplate shaker at ~600rpm.

6. Aspirate each well and wash, repeating the process 3 times for a **total 4 washes**. Wash by filling each well with **1× Wash Buffer (350 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting.
   (Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.)
7. Add **100 µl** of the **1X Antibody conjugate working solution** into all wells. Gently tap the plate to mix well. Cover the plate and **incubate for 1 h at 37°C** on a microplate shaker at ~600rpm.
8. Aspirate each well and **wash as step 6**.
9. Add **100 µl** of the **1X HRP-Streptavidin working solution** into all wells. Gently tap the plate to mix well. Cover the plate and **incubate for 30 min at room temperature (20-25°C)**.
10. Aspirate each well and **wash as step 6**.
11. Add **100 µl** of **TMB substrate solution** into each well. **Incubate for 30 mins at RT** without shaking. Avoid exposure to light.
12. Add **100 µl** of **Stop Solution** to each well and shake or tap the plate lightly to ensure homogeneous mixing. The color of the solution should change from blue to yellow.
13. Read the OD with a microplate reader at **450 nm** immediately. It is recommended read the absorbance within 30 min after adding Stop solution.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

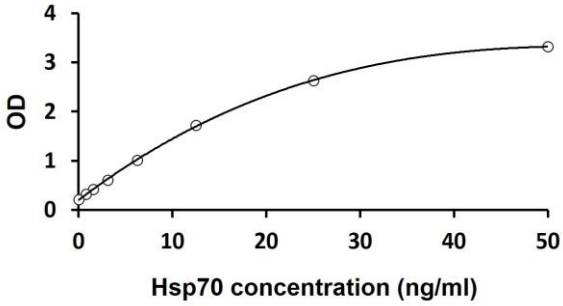
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

5. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)

6. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above. If the samples are following the suggested sample preparation dilution and assay procedure, the dilution factor would be 8 (dilution ratio (4) * Pre-Treatment Buffer dilution (2) = 8).

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

The minimum detectable dose (MDD) of HSP70 ranged from 0.781-50 ng/ml. The mean MDD was 0.18 ng/ml.

Specificity

This ELISA kit is specific for Hsp70. This HSP70 ELISA kit has been validated for the detection of Hsp70 from Human, Dog and Goat.