HSP70 ELISA Kit

Cat #: orb1822522 (manual)

GENERAL INFORMATION

Materials Supplied

Item	Quantity/Size
Anti-Hsp70 Immunoassay Plate	1 Plate
Recombinant Hsp70 Standard	2 vials
Standard and Sample Diluent (Red)	1 vial/50mL
10X Wash Buffer Concentrate	1vial/100mL
Anti-Hsp70 Biotinylated Antibody Concentrate	1 vial/150µL
Anti-Hsp70 Biotinylated Antibody Diluent (Green)	1 vial/13mL
Streptavidin HRP Concentrate	1 vial/50µL
Streptavidin HRP Diluent (Purple)	1 vial/13mL
TMB Substrate	1 vial/13mL
Stop Solution	1 vial/13mL

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

Storage and Stability

All reagents with the exception of the standard and Streptavidin HRP Concentrate are stable as supplied at 4°C, the standard and Streptavidin HRP Concentrate should be stored at -20°C. Unused wells should be resealed with desiccant in the foil pouch provided, and stored at 4°C until the kit's expiry date.

Materials Needed but Not Supplied

- Ultra-pure water
- Additional reagents and materials for cell lysate and tissue extract preparation, including protease inhibitors
- Precision pipettors, with disposable plastic tips

- Polypropylene or polyethylene tubes to prepare samples − do not use polystyrene, polycarbonate or glass tubes
- A container to prepare 1X Wash Buffer
- A wash bottle or an automated 96-well plate washer
- Disposable reagent reservoirs
- A standard microtiter plate reader for measuring absorbance at 450 nm
- Adhesive plate sealers
- Plate shaker/incubator

Assay Precautions

- All ELISA reagents must be at room temperature (20-25°C) before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Take care not to contaminate the TMB Substrate. Do not expose TMB Substrate solution to glass, foil, or metal. If the solution is blue before use, DO NOT USE IT.
- Individual components may contain preservatives. Wear gloves while performing the assay. Please follow proper disposal procedures.

INTRODUCTION

Background

Hsp70 genes encode abundant heat-inducible 70-kDa hsps (hsp70s). In most eukaryotes hsp70 genes exist as part of a multigene family. They are found in most cellular compartments of eukaryotes including nuclei, mitochondria, chloroplasts, the endoplasmic reticulum and the cytosol, as well as in bacteria. The genes show a high degree of conservation, having at least 5O% identity (2). The N-terminal two thirds of hsp70s are more conserved than the C-terminal third. Hsp70 binds ATP with high affinity and possesses a weak ATPase activity which can be stimulated by binding to unfolded proteins and synthetic peptides (3). When hsc70 (constitutively expressed) present in mammalian cells was truncated, ATP binding activity was found to reside in an N-terminal fragment of 44kDa which lacked peptide binding capacity. Polypeptide binding ability therefore resided within the C-terminal half (4). The structure of this ATP binding domain displays multiple features of nucleotide binding proteins (5). All hsp70s, regardless of location, bind proteins, particularly unfolded ones. The molecular chaperones of the hsp70 family recognize and bind to nascent polypeptide chains as well as partially folded intermediates of proteins preventing their aggregation and misfolding. The binding of ATP triggers a critical conformational change

leading to the release of the bound substrate protein (6). The universal ability of hsp70s to undergo cycles of binding to and release from hydrophobic stretches of partially unfolded proteins determines their role in a great variety of vital intracellular functions such as protein synthesis, protein folding and oligomerization and protein transport.

About This Assay

ELISA Kit is for the detection of Human and cross-reactive sample types of Hsp70 in plasma, serum and cell lysates.

Each kit contains sufficient components to quantitate the Hsp70 concentration in up to 40 samples, tested in duplicate.

Western Blot Validation of Capture Antibody

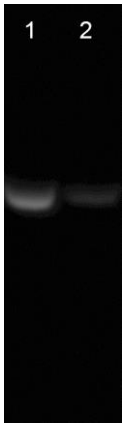

Figure 1: Western blot analysis of Hsp70.

Lane 1- 7.5µg of SKBR3 lysate (Human)

Lane 2- 7.5µg of MDCK lysate (Dog)

ASSAY OVERVIEW

1. Prepare Standard and samples in Standard and Sample Diluent.
2. Add 100 µL of Standard or sample to appropriate wells.
3. Cover plate with Plate Sealer and incubate at 37°C for 1 hour with shaking at 600rpm.
4. Wash plate four times with 1X Wash Buffer.
5. Add 100 µL of Biotinylated Antibody Working Solution to each well.
6. Cover plate with Plate Sealer and incubate at 37°C for 1 hour with shaking at 600rpm
7. Wash plate four times with 1X Wash Buffer.
8. Add 100 µL of Streptavidin HRP Working Solution to each well.
9. Cover plate with Plate Sealer and incubate at 37°C for 30 minutes with shaking at 600rpm.
10. Wash plate four times with 1X Wash Buffer.

11. Add 100 µL of TMB Substrate to each well.
12. Develop the plate in the dark at room temperature for 30 minutes.
13. Stop reaction by adding 100 µL of Stop Solution to each well.
14. Measure absorbance on a plate reader at 450 nm.

PRE-ASSAY PREPARATION

Sample Preparation

Cell Lysate Preparation

1. Prepare and treat cells as desired.
2. 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. 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 (3) 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 -70ºC and lysed at a later time, if desired.
5. Calculate the amount of Extraction Reagent required (Not Supplied). Appropriate Extraction Reagents must contain ≤0.1% SDS eg. 50mMTris ph7.2, 150 mM NaCl, 1% Triton X-100. For every 1 X 106 to 1X 107 cells, use 1 mL of 1X Extraction Reagent.

Note: Alternative extraction reagents may be used, but reagents containing more than 0.1% SDS will affect results.

6. Add protease inhibitors to the 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 pep statin. 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 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 sonification.

Note: To increase proteinyields and decrease sample viscosity, aspirate the cellpellet 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 4ºC.
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 -70ºC. It is recommended that a protein determination assay be performed and the extracts aliquoted into convenient amounts prior to storing at -70ºC to avoid multiple freeze-thaw cycles.

Tissue Extract Preparation

1. Harvest tissue to be analyzed. Tissues my be flash frozen, stored at -70ºC and prepared at a later time, if desired.
2. Calculate the amount of Extraction Reagent required (Not Supplied). Appropriate Extraction Reagents must contain ≤0.1% SDS eg. 50mMTris ph7.2, 150 mM NaCl, 1% Triton X-100. For each 0.5 cm3 piece of tissue, use 1 mL of Extraction Reagent.

Note: Alternative extraction reagents may be used, but reagents containing more than 0.1% SDS will affect results.

3. Add protease inhibitors to the 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 pep statin. 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. appropriate amount of ice-cold Extraction Reagent including protease inhibitors to the processed tissue.
8. 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 4ºC.
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 -70ºC. It is recommended that a protein determination assay be performed and the extracts aliquoted into convenient amounts prior to storing at -70ºC to avoid multiple freeze-thaw cycles.

Serum Collection

1. Collect whole blood using established methods.
2. Allow samples to clot at room temperature for 30 minutes.
3. Centrifuge at 2400 x g for 10 minutes, taking precautions to avoid hemolysis.
4. Transfer the serum to a fresh tube. The serum collected is now ready for analysis in the assay.
5. Alternatively, store serum samples in single-use aliquots at <-20ºC. Avoid multiple freeze-thaw cycles.

Plasma Collection

1. Collect whole blood using established methods in K2 EDTA, Sodium Heparin or Sodium Citrate anticoagulant.
2. Centrifuge at 2400 x g for 10 minutes, taking precautions to avoid hemolysis.
3. Transfer the cell-free plasma to a fresh tube. The plasma collected is now ready for analysis in the assay.

4. Alternatively, store plasma samples in single-use aliquots at ≤-20ºC. Avoid multiple freeze-thaw cycles.

Sample Handling

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.
- 100 µl of diluted sample is required per well.
- Samples must be assayed in duplicate each time the assay is performed.
- Samples should be frozen if not analyzed shortly after harvest. For long-term storage, aliquot and freeze samples. Avoid repeated freeze-thaw cycles when storing samples.
- If particulate is present in samples, centrifuge prior to analysis.
- If the integrity of the sample is of concern, make a note on the Plate Template and interpret results with caution.

Sample Dilution

- Samples must first be diluted prior to testing.
- Suggested starting dilutions for samples:

✧ For native human cell lysates, dilute samples 1:128 in Standard and Sample Diluent. For example, dilute 2 µL of sample in 254 µL Standard and Sample Diluent. Mix well. For non-human cell lysates, dilute samples 1:4 in Standard and Sample Diluent.

✧ For serum and plasma samples, dilute samples 1:4 in Standard and Sample Diluent. For example, dilute 60 µL of sample in 180 µL Standard and 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 240 µL of sample in Standard and Sample Diluent. Mix samples well prior to analysis.

Note: The cell lysate Extraction Reagent is not included with this kit. This kit is mainly for use with serum and plasma samples.

Other Reagent Handling/ Preparation

Standard Preparation

1. Reconstitute standard vial with 0.85 mL of Standard and Sample Diluent for a concentration of 27 ng/mL. Mix well.
2. Label seven (7) tubes, one for each additional standard curve point: 13.5 ng/mL, 6.75 ng/mL, 3.38 ng/mL, 1.69 ng/mL, 0.84 ng/mL, 0.42 ng/mL, and 0 ng/mL.
3. Pipet 250 µl of Standard and Sample Diluent into each tube.
4. Serial dilute the 27 ng/mL standard 1:1 with Standard and Sample Diluent. Perform dilution by mixing 250 µL of the previous standard with 250 µL of Standard and Sample Diluent. Continue until reach the standard value of 0.42 ng/mL.
5. Use Standard and Sample Diluent only as the zero-standard value.

6. For non-human and monkey samples, it is recommended to substitute the provided standard Human Hsp70 with a protein standard that is directly relevant to the sample species.

1X Wash Buffer Preparation

- Prepare 1X Wash buffer by diluting 10X Wash Buffer in ultra-pure water. For example, if preparing 1 L of 1X Wash Buffer, dilute 100 mL of 10XWash Buffer into 900 mL of ultra-pure water. Mix well. Store reconstituted 1X Wash Buffer at 2-8ºC for up to one (1) month. Do not use 1X Wash Buffer if it becomes visibly contaminated during storage.

Biotinylated Antibody Working Solution Preparation

- Determine the amount of Biotinylated Antibody Working Solution required. For every strip-well used (8-wells), prepare 1 mL of Biotinylated Antibody Working Solution.
- Prepare Biotinylated Antibody Working Solution by diluting Biotinylated Antibody Concentrate 1:100 with Biotinylated Antibody Diluent. For example, if 12 mL of Biotinylated Antibody Working Solution is required (one whole plate), dilute 120 µL of Biotinylated Antibody Concentrate in 12 mL Biotinylated Antibody Diluent. Mix well prior to use.

Streptavidin HRP Working Solution Preparation

- Determine the amount of Streptavidin HRP Working Solution required. For every strip-well used (8-wells), prepare 1 mL of Streptavidin HRP Working Solution.
- Prepare Streptavidin HRP Working Solution by diluting Streptavidin HRP Concentrate 1:100 with Streptavidin HRP Diluent. For example, if 12 mL of Streptavidin HRP Working Solution is required (one whole plate), dilute 120 µL of Streptavidin HRP Concentrate in 12 mL Streptavidin HRP Diluent. Mix well prior to use.

ASSAY PROTOCOL

Sample Incubation

- Determine the number of strips required. Leave these strips in the plate frame. Place unused strips in the foil pouch with desiccant and seal tightly. Store unused strips at 2-8°C. After completing assay, keep the plate frame for additional assays.
- Use a Plate Template to record the locations of the standards and unknown samples within the wells.

1. Add 100 µL of appropriately diluted standards or samples to each well. Run each standard, sample, or blank in duplicate.
2. Carefully cover wells with a new adhesive plate cover. Incubate for one (1) hour at 37°C with shaking at 600rpm.
3. Carefully remove adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer as described in the Plate Washing section.

Plate Washing

1. Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.
2. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure three additional times for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate plate contents from all wells and flood wells with 1X Wash Buffer. Repeat procedure three additional times for a total of FOUR washes. Additional washes may be necessary. Blot plate onto paper towels or other absorbent material.

Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Biotinylated Antibody Incubation

- Prepare only the required amount of Biotinylated Antibody Working Solution for the number of strips being used.

1. Add 100 µL of Biotinylated Antibody Working Solution to each well containing standard, sample or blank. Mix well by gently tapping the plate several times.
2. Carefully attach a new adhesive plate cover. Incubate plate for one (1) hour at 37°C with shaking at 600rpm.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer as described in the Plate Washing section.

Streptavidin HRP Incubation

- Prepare only the required amount of Streptavidin HRP Working Solution for the number of strips being used.

1. Add 100 µL of Streptavidin HRP Working Solution to each well containing standard, sample or blank.
2. Carefully attach a new adhesive plate cover. Incubate plate for 30 minutes at 37°C with shaking at 600rpm.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer as described in the Plate Washing section.

TMB Substrate Incubation and Reaction Stop

- Only remove the required amount of TMB Substrate and Stop Solution for the number of strips being used.
- Do NOT use a glass pipette to measure the TMB Substrate solution. Do NOT cover the plate with aluminium foil or metalized mylar. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate. If the solution is blue before use, DO NOT USE IT!

1. Add 100 µL of TMB Substrate into each well.
2. Allow the enzymatic color reaction to develop at room temperature (20-25ºC) in the dark for 30 minutes. Do NOT cover plate with a plate sealer. The substrate reaction yields a blue solution.
3. After 30 minutes, stop the reaction by adding 100 µL of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should change from blue to yellow.

Absorbance Measurement

Note: Evaluate the plate within 30 minutes of stopping the reaction.

1. Wipe underside of wells with a lint-free tissue.
2. Measure the absorbance on an ELISA plate reader set at 450 nm.

ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively, a spreadsheet program can be used.

Calculations

- Duplicate absorbance values should be within 10% of each other. Care should be taken when interpreting data with differences in absorbance values greater than 10%.

1. Prepare a standard curve to determine the amount of Hsp70 in an unknown sample. Plot the average absorbance obtained for each standard concentration on the vertical (Y) axis versus the corresponding Hsp70 concentration on the horizontal (X) axis using graph paper or curve-fitting software.
2. Calculate the Hsp70 concentration in unknown samples using the prepared standard curve. Determine the amount of Hsp70 in each unknown sample by noting the Hsp70 concentration (X axis) that correlates with the absorbance value (Y axis) obtained for the unknown sample.
3. Multiply the Hsp70 concentration obtained by the dilution factor to determine the amount of Hsp70 in the undiluted sample.

Performance Characteristics

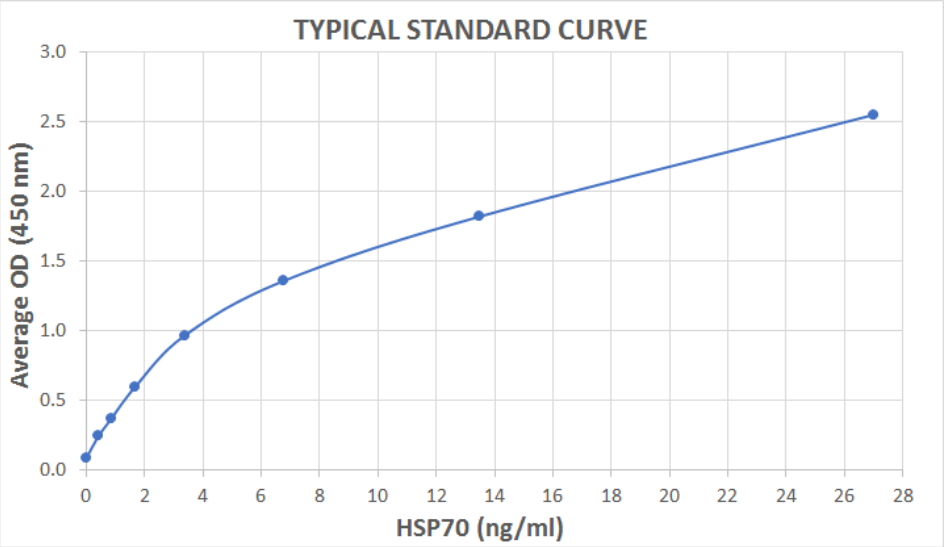

Figure 2. Typical standard curve

Note: This typical standard curve was generated using the Hsp70 ELISA Kit Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay.

Assay Range: 0.42-27 ng/mL

- Suggested standard curve points are 27 ng/mL, 13.5 ng/mL, 6.75 ng/mL, 3.38 ng/mL, 1.69 ng/mL, 0.84 ng/mL, 0.42 ng/mL, and 0 ng/mL.

Assay Specificity and Species Reactivity

- This assay is specific for Hsp70.
- The Hsp70 ELISA has been validated for the detection of Human, Monkey, Canine, Rat, and Mouse Hsp70.

Sensitivity

- The calculated sensitivity of the Hsp70 ELISA is 0.038 ng/mL.

Assay Limitations

- This assay has been validated for use with plasma, serum, 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. Cell lysates can be screened as long as the SDS contect is 0.1% or lower.
- Some samples may contain higher levels of interfering factors that can produce abnormal results.
- If values for samples are not within the range of the standard curve, optimal sample dilutions need to be determined.
- The use of assay reagents not provided in this kit can compromise the performance of this assay.
- Do not mix components with reagents from other kits with different lot numbers.
- For non-human and monkey samples, it is recommended to substitute the provided standard Human Hsp70 with a protein standard that is directly relevant to the sample species.

Warranty and Limitation of Remedy

Biorbyt makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Biorbyt **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Biorbyt will carry out its delivery obligations with due care and skill. Thus, in no event will Biorbyt have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Biorbyt is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Biorbyt, its directors or its employees.