



**ab124534 –**

**HSP60 (HSPD1)**

**Human ELISA Kit**

## Instructions for Use

For the quantitative measurement of Human HSP60 protein

This product is for research use only and is not intended for diagnostic use.



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# 1. Introduction

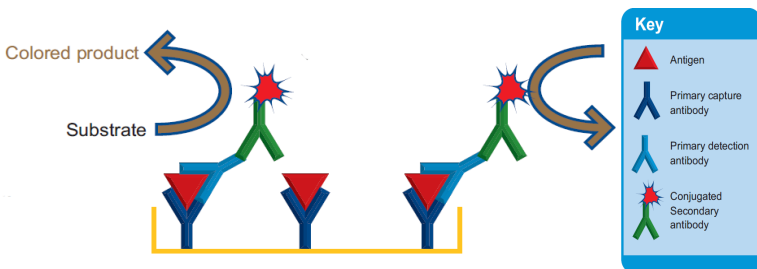
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**Principle:** ab124534 HSP60 (HSPD1) Human ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of HSP60 protein a 60 kDa protein encoded by the Human HSPD1 gene, in cell and tissue lysates. The assay employs an HSP60 specific antibody coated onto well plate strips. Standards and samples are pipetted into the wells and analyte present in the sample is bound to the wells by the immobilized antibody. The wells are washed and an anti-HSP60 primary detector antibody is added. After washing away unbound primary detector antibody, HRP-conjugated secondary detector antibody specific for the primary detector antibody is pipetted to the wells. The wells are again washed, a HRP solution is added to the wells and color develops in proportion to the amount of analyte bound. The developing blue color is measured at 600 nm. Optionally the reaction can be stopped by adding hydrochloric acid which changes the color from blue to yellow and the intensity can be measured at 450 nm.

**Background:** HSP60 (gene HSPD1) is a mitochondrial matrix protein implicated in protein import from the cytoplasm into the mitochondrion and macromolecular assembly. HSP60 is a chaperone and so facilitates the correct folding of imported proteins. HSP60 prevent misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress

conditions in the mitochondrial matrix. HSP60 has been implicated in diabetes, stress response, certain immunological disorders and cancer. In several studies of carcinomas decreased HSP60 levels correlated with poor prognosis.

Specifically defects in HSPD1 are a cause of spastic paraplegia autosomal dominant type 13 (SPG13). Spastic paraplegia is a degenerative spinal cord disorder characterized by a slow, gradual, progressive weakness and spasticity of the lower limbs. Defects in HSPD1 are the cause of leukodystrophy hypomyelinating type 4 (HLD4); also called mitochondrial HSP60 chaperonopathy or MitCHAP-60 disease. HLD4 is a severe autosomal recessive hypomyelinating leukodystrophy. Clinically characterized by infantile-onset rotary nystagmus, progressive spastic paraplegia, neurologic regression, motor impairment, profound mental retardation. Death usually occurs within the first two decades of life.



## 2. Assay Summary:

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Equilibrate all reagents to room temperature. Prepare all the reagents, samples, and standards (optional, not included in the kit)

as instructed.



Add 50  $\mu$ L standard (optional, not included in the kit) or sample to each well used. Incubate 2 hours at room temperature.



Aspirate and wash each well two times. Add 50  $\mu$ L prepared 1X Detector Antibody to each well. Incubate 1 hour at room temperature.



Aspirate and wash each well two times. Add 50  $\mu$ L prepared 1X HRP Label. Incubate 1 hour at room temperature.



Aspirate and wash each well three times. Add 50  $\mu$ L HRP Development Solution to each well. Immediately begin recording the color development for 15 minutes at 600 nm. Alternatively add a Stop solution at a user-defined time and read at 450 nm.

### 3. Kit Contents

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<b>Item</b>	<b>Quantity</b>
Extraction Buffer	15 mL
20X Buffer	20 mL
10X Blocking Buffer	6 mL
HSP60 Microplate (8 x 12 antibody coated strips)	1
10X HSP60 Detector Antibody	1 mL
10X HRP Label	1 mL
HRP Development Solution	12 mL

### 4. Storage and Handling

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Store all components at 4°C. This kit is stable for at least 6 months from receipt. Unused microplate strips should be returned to the pouch containing the desiccant and resealed.

## 5. Additional Materials Required

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- Microplate reader capable of measuring absorbance at 600 nm (or 450 nm after addition of Stop solution - not supplied).
- Method for determining protein concentration (BCA assay recommended).
- Deionized water
- Multi and single channel pipettes
- PBS (1.4 mM  $\text{KH}_2\text{PO}_4$ , 8 mM  $\text{Na}_2\text{HPO}_4$ , 140 mM NaCl, 2.7 mM KCl, pH 7.3)
- Tubes for sample dilution
- Stop solution (optional) – 1N hydrochloric acid
- Optional plate shaker for all incubation steps
- Microplate cover or seals

## 6. Preparation of Reagents

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- 6.1 Equilibrate all reagents to room temperature (18-25°C) before use.
- 6.2 Prepare 1X Wash Buffer by adding 20 mL 20X Buffer to 380 mL nanopure water. Mix gently and thoroughly.
- 6.3 Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Solution to 54 mL 1X Wash Buffer. Unused 1X Incubation

Buffer may be stored at -20°C for 6 months after performing the ELISA.

6.4 Prepare the 1X HSP60 Detector Antibody by diluting the 10X HSP60 Detector Antibody 10-fold with 1X Incubation Buffer immediately prior to use. Prepare 0.5 mL for each 8 well strip used.

6.5 Prepare the HRP Label by diluting the stock 10X HRP Label 10-fold with 1X Incubation Buffer immediately before use. Prepare 0.5 mL for each 8 well strip used.

## 7. Sample Preparation

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**Note: Extraction buffer can be supplemented with phosphatase inhibitors, PMSF and protease inhibitor cocktail prior to use. Supplements should be used according to manufacturer's instructions.**

### 7.1 Preparation of extract from cell pellets

7.1.1 Collect non adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 10 min at 4°C.

7.1.2 Rinse cells twice with PBS.

7.1.3 Solubilize cell pellet at  $2 \times 10^7$ /mL in Extraction Buffer.

7.1.4 Incubate on ice for 20 minutes. Centrifuge at 16,000 x g for 20 minutes at 4°C. Transfer the

supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at  $-80^{\circ}\text{C}$  for 6 months. The sample protein concentration in the extract may be quantified using a protein assay.

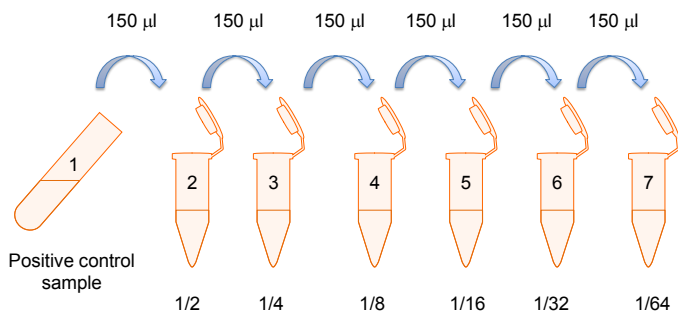
## **7.2 Preparation of extracts from tissue homogenates**

- 7.2.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).
- 7.2.2 Suspend the homogenate to 25 mg/mL in PBS.
- 7.2.3 Solubilize the homogenate by adding four volumes of Extraction Buffer to a final sample protein concentration of 5 mg/mL.
- 7.2.4 Incubate on ice for 20 minutes. Centrifuge at 16,000 x g, for 20 minutes at  $4^{\circ}\text{C}$ . Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at  $-80^{\circ}\text{C}$ . The sample protein concentration in the extract may be quantified using a protein assay.

### 7.3 Positive control sample dilution series preparation

**Note: It is strongly recommended to prepare a dilution series of a standard control sample (not included in this kit). The reference standard curves of several human cell lines can be found in section 8 data analysis.**

- 7.3.1 To prepare serially diluted positive control sample, label six tubes #2-7.
- 7.3.2 Prepare a positive control sample lysate or extract as directed in previous sections (6.1, 6.2). Dilute the positive control sample to an upper concentration limit of the working range of the assay in the same diluent used to dilute other experimental samples. Label this tube #1.
- 7.3.3 Add 150  $\mu\text{L}$  of the diluent to each of tubes #2 through #7.
- 7.3.4 Transfer 150  $\mu\text{L}$  from tube #1 to tube #2. Mix thoroughly. With a fresh pipette tip transfer 150  $\mu\text{L}$  from #2 to #3. Mix thoroughly. Repeat for Tubes #4 through #7. Use the diluent as the zero standard tube labeled #8. Use fresh control sample dilutions for each assay.



## 8. Assay Procedure

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**Equilibrate all reagents and samples to room temperature before use. It is recommended all samples and controls be assayed in duplicate.**

- 8.1 Prepare all reagents, working dilutions of controls and samples as directed in the previous sections.
- 8.2 Remove unused microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and re-seal.
- 8.3 Add 50 µL of each sample per well. It is recommended to include a dilution series of a positive control sample (section 6.3). Also include a no material control (diluent) as a zero standard.

- 8.4 Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
- 8.5 Aspirate each well and wash, repeat this once more for a total of **two** washes. Wash by aspirating or decanting from wells then dispensing 300  $\mu$ L 1X Wash Buffer into each well as described above. Complete removal of liquid at each step is essential to good performance. After the last wash, remove the remaining buffer by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.
- 8.6 Immediately prior to use prepare sufficient (0.5 mL/strip used) 1X HSP60 Detector Antibody (step 5.4) in 1X Incubation Buffer. Add 50  $\mu$ L 1X HSP60 Detector antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
- 8.7 Repeat the aspirate/wash procedure above.
- 8.8 Immediately prior to use prepare sufficient (0.5 mL/strip used) 1X HRP Label in 1X Incubation Buffer (step 5.5). Add 50  $\mu$ L 1X HRP Label to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
- 8.9 Repeat the aspirate/wash procedure above, however, performing a total of **three** washes.

8.10 Add 50  $\mu\text{L}$  HRP Development Solution to each empty well and immediately record the blue color development with elapsed time in the microplate reader prepared with the following settings:

<b>Mode:</b>	<b>Kinetic</b>
Wavelength:	600 nm
Time:	up to 15 min.
Interval:	20 sec. - 1 min.
Shaking:	Shake before and between readings

*Alternative*— In place of a kinetic reading, at a **user defined**, time record the endpoint OD data at (i) 600 nm or (ii) stop the reaction by adding 50  $\mu\text{L}$  stop solution (1N HCl) to each well and record the OD at 450 nm.

8.11 Analyze the data as described below.

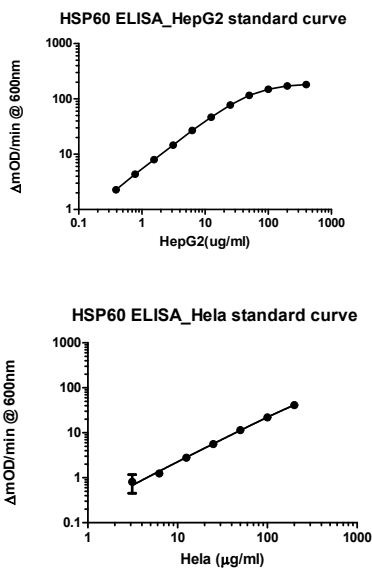
## 9. Data Analysis

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Average the duplicate positive controls readings and plot against their concentrations after subtracting the zero standard reading. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-

log, log/log, 4 parameter logistic). Read relative HSP60 concentrations for unknown samples from the standard curve plotted (for example see Figure 1). Samples producing signals greater than that of the highest standard should be further diluted in and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

**TYPICAL STANDARD CURVE - *For demonstration only.***



*Figure 1.* Example standard curves. (Upper) A dilution series of HepG2 cell lysate in the working range of the assay. (Lower) A dilution series of HeLa cell lysate in the working range of the assay.

## TYPICAL WORKING RANGES FOR PELLET EXTRACTS

Sample Type	Range
HepG2 cell extract	0.39 - 200 $\mu\text{g/mL}$
Hela cell extract	3.125 - 200 $\mu\text{g/mL}$

## LINEARITY OF DILUTION

Linearity of dilution determined by comparing dilution series of extracts prepared from HepG2 cell pellets (starting extract concentration is 50 $\mu\text{g/mL}$ )

HepG2 ( $\mu\text{g/ml}$ )	% Expected Value
1:1	71.8
1:2	86.6
1:4	100
1:8	108.5
1:16	118.5
1:32	129.44
1:64	134.94

## REPRODUCIBILITY

Parameter	CV%
Intra (n=4)	2.1
Inter (n=3)	6.7

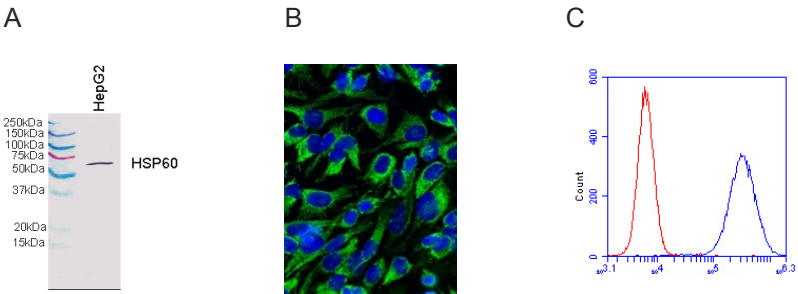
## RECOVERY

HepG2 ( $\mu\text{g/ml}$ )	CONTROL	10% goat serum	50% culture medium	50% incubation buffer
25	100	79	99	102
12.5	100	76	97	88
6.25	100	77	95	91
3.125	100	76	95	91
1.56	100	75	93	92
0.78	100	77	93	89
0.39	100	75	87	87

## 10. Specificity

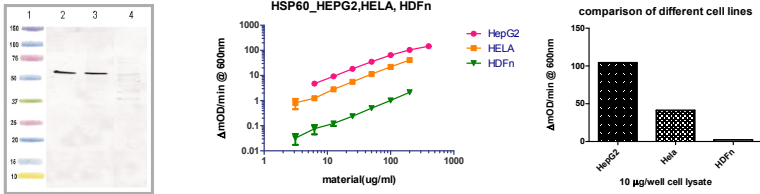
Species– Human reactive. Non-reactive with mouse and rat. Others untested.

The detector antibody in this assay specifically reacts with the 60 kDa HSP60 as shown in Figure 2.



**Figure 2.** Demonstration the specificity of antibodies used in this kit. (A) Western blot using the HSP60 detector antibody at 1  $\mu\text{g}/\text{mL}$ : HepG2 cell lysate, HeLa cell lysate, and HDFn lysate, each were loaded at 15  $\mu\text{g}/\text{lane}$ . (B) Analysis of HSP60 staining in HDFn cells by ICC: HSP60 primary antibody at 1  $\mu\text{g}/\text{mL}$  (green). (C) Analysis of HSP60 staining in Hela cells by Flow: blue: 1 $\mu\text{g}/\text{mL}$  HSP60 antibody; red: an equal amount of an isotype control antibody.

HSP60 is expressed at different levels in human cultured cell lines as indicated by Western blot and determined using this HSP60 ELISA assay shown in Figure 3.



*Figure 3.* Demonstration of differential HSP60 expression in human cell lysates. (A) HSP60 levels are indicated by Western blot in HepG2 cell lysate (lane 2), HeLa cell lysate (lane 3), and HDFn lysate (lane 4). Each sample was loaded at 15  $\mu$ g/lane. (B) Endogenous HSP60 levels were detected in different cell lysates using HSP60 ELISA kit. The HSP60 ELISA kit shows greater sensitive and accuracy to demonstrate the quantitative relationship among different human cell lines.

## 11. Troubleshooting

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<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
Poor standard curve	Inaccurate Pipetting	Check pipets
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Low HSP60 concentration in sample	Use appropriate positive control
	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep substrate solution protected from light







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