

FOR RESEARCH USE ONLY

NOT FOR IN VITRO CLINICAL DIAGNOSIS

EasyPro Rat Heat Shock 70kDa Protein 4(HSPA4)ELISA Kit

Catalog NO.:FY-ER3431S size: 96T/48T

This manual must be read attentively and completely before using this product.
If you have any problems, please contact us.

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Intended Use

The kit is intended for use in quantitative determination of Rat HSPA4 concentrations in serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

Specification

- ★ Sensitivity:0.1 ng/mL.
- ★ Detection Range:0.16-10ng/mL.
- ★ No significant cross-reactivity or interference between Rat HSPA4 and analogues was observed.
- ★ Repeatability: Coefficient of variation is 8%.

Principle of the Procedure

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat HSPA4. Samples (or Standards) and and Horseradish Peroxidase (HRP) linked antibody specific for Rat HSPA4 are added to the micro ELISA plate wells. Rat HSPA4 in samples (or standards) combines with the coated antibody and HRP linked detection antibody special to Rat HSPA4. Excess conjugate and unbound sample or standard are washed from the plate. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of Rat HSPA4. You can calculate the concentration of Rat HSPA4 in the samples by comparing the OD of the samples to the standard curve.

Limitations of the Procedure

1. This kit is for laboratory scientific research only, we will not be responsible for any consequences if this kit is used for clinical diagnosis or any other procedures.
2. Due to the uncertainty of its validity, this kit may not be suitable for testing some special experimental samples, such as gene knockout experiments.
3. This kit should be used before its expiration date, and please strictly follow the instructions for storage.
4. Different manufacturers' kits or testing the same analyte by other methods may produce inconsistent results because we do not compare our products with those of other manufacturers.
5. Since the antibodies used in the kit are usually prepared from recombinant proteins as immunogen, and recombinant proteins can be limited by different fragmentation, expression and purification systems, it is not recommended to use this kit to detect recombinant proteins
6. In order to get the best experimental results, please use only the reagents

- provided by the manufacturer, and do not mix reagents from different batches.
7. Due to the existing conditions and the limitations of science and technology, we cannot fully identify and analyze the raw materials provided by the supplier comprehensively. Therefore, the kit may have some quality and technical risks.
 8. The possibility of interference cannot be excluded before all factors are tested in the ELISA immunoassay.
 9. In order to obtain reproducible results, each step in the experiment should be controlled and variations in sample collection, handling and storage may also lead to differences in sample measurements.
 10. Although each kit passes rigorous quality testing, differences in measured values between batches of kits can still be caused by factors such as shipping conditions and different laboratory equipment.

Reagents&Materials Provided

| Item | Specifications | Storage |
|--|---|----------------------------|
| Micro ELISA Plate (Dismountable) | 96T: 8 wells ×12 strips 48T: 8 wells ×6 strips 24T: 8 wells ×3 strips | -20°C, 6 months |
| Reference Standard | 96T: 2 vials 48T: 1 vial 24T: 1 vial | |
| Concentrated HRP Conjugate (100×) | 96T: 1 vial, 60μL 48T: 1 vial, 30μL 24T: 1 vial, 30μL | -20°C, 6 months |
| Reference Standard & Sample Diluent | 1 vial, 20 mL | 2-8°C, 6 months |
| HRP Conjugate Diluent | 1 vial, 13mL | |
| Concentrated Wash Buffer (25×) | 1 vial, 30 mL | |
| Substrate Reagent | 1 vial, 10 mL | 2-8°C (Protect from light) |
| Stop Solution | 1 vial, 10 mL | 2-8°C, 6 months |

| | | |
|-------------------------|----------|----|
| Plate Sealer | 5 pieces | RT |
| Product Description | 1 copy | |
| Certificate of Analysis | 1 copy | |

Materials & Equipment Required But Not Provided

Microplate reader with 450nm wavelength filter

Incubator capable of maintaining 37°C

Single or multi-channel pipettes with high precision
 disposable pipette tips

EP tubes

Container for Wash Solution

Squirt bottle, manifold dispenser, or automated microplate washer

Deionized or distilled water

Absorbent paper for blotting the microplate

Sample collection

Serum: Allow blood samples to clot for 2 hours at room temperature or overnight at 2-8°C before centrifugation for 15 min at 2000×g at 2-8°C. Supernatants should be taken for assay testing immediately or stored at -20°C or -80°C for later use.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 2000×g at 2-8°C within 30 min of collection. Supernatants should be taken for assay testing immediately or stored at -20°C or -80°C for later use.

Tissue homogenates: Tissues should be rinsed in ice-cold PBS to remove excess blood thoroughly, weighed, minced into small pieces. then Tissue pieces omogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. The resulting suspension is sonicated with an ultrasonic cell disrupter till the solution is clarified. The homogenates are then centrifuged for 5

min at 10000×g. Supernatants should be taken for assay testing immediately or stored at -20°C or -80°C for later use.

Cell lysates: For adherent cells, gently wash the cells with moderate amount of cool PBS and detach the cells with trypsin. Collect cells by centrifugation for 5 min at 1500×g(suspension cells can be collected by centrifugation directly). Discard the supernate and wash cells 3 times with cool PBS. Resuspend cells in cool PBS with concentration of 5×10^6 cells/mL. Repeat the freeze-thaw process several times until the cells are fully lysed. Centrifuge for 15min at 2000×g at 2-8°C. Supernatants should be taken for assay testing immediately or stored at -20°C or -80°C for later use.

Cell culture supernatant and other biological fluids: Centrifuge samples for 15 min at 1500× g at 2-8°C. Supernatants should be taken for assay testing immediately or stored at -20°C or -80°C for later use.

Note for sample

1. Samples should be used within 6 days when stored at 2-8°C, otherwise samples must be stored at -20°C (≤ 1 month) or -80°C (≤ 2 months). Avoid repeated freeze-thaw cycles.
2. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
4. If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant, there is a possibility to cause a deviation due to the introduced chemical substance.
5. Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.
6. Please do not use hemolyzed samples for ELISA as it will affect the test results.
7. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong result.

Reagent Preparation

1. Allow all reagents to return to room temperature (18-25°C) before use.
2. **Wash Buffer:** Dilute 20 mL of Concentrated Wash Buffer with 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.
3. **Standard working solution:** First, centrifuge the standard at 1000×g for 1 min, add 1mL of standard sample dilution, let it stand for 10min and then mix gently, that is 10ng/mL of standard working solution. Second, take 7 EP tubes, add 500μL of Standard & Sample Diluent to each tube. Pipette 500μL of the 10ng/mL working solution to the first tube and mix up to produce a 5ng/mL working solution. Pipette 500μL of the solution from the former tube into the latter one according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.63ng/mL, 0.32ng/mL, 0.16ng/mL and the last EP tubes with Standard & Sample Diluent is the blank as 0ng/mL



| | | | | | | | |
|-------|-------|-------|-------|-------|-------|-------|-------|
| 10 | 5 | 2.5 | 1.25 | 0.63 | 0.32 | 0.16 | 0 |
| ng/mL | ng/mL | ng/mL | ng/mL | ng/mL | ng/mL | ng/mL | ng/mL |

4. HRP Conjugate working solution: Calculate the required amount before the experiment (50 μ L/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated HRP Conjugate at 800 \times g for 1 min, then dilute the 100 \times Concentrated HRP Conjugate to 1 \times working solution with HRP Conjugate Diluent (Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99).

Assay Protocol

1. The Micro ELISA Plate slats to be used were removed from the plate frame and the remaining slats were returned to the aluminum foil bag containing the desiccants and then resealed for storage.
2. Determine wells for diluted standard, blank and sample. Add 50 μ L each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate). Immediately add 50 μ L of HRP Conjugate working solution to each well. Cover the plate with a new sealer. Incubate for 60 min at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
3. Decant the solution from each well add 300 μ L of wash buffer to each well. Soak for 0.5 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.
4. Add 100 μ L of Substrate Reagent to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.
5. Add 50 μ L of Stop Solution to each well. Note: adding the stop solution should be done in the same order as the substrate solution.
6. Determine the optical density (OD value) of each well at once with a micro plate reader set to 450 nm.

Example Data

The OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), so plotting log of the data to establish a standard curve for each test is strongly recommended. Typical standard curve is provided below for reference only.

| Concentration (ng/mL) | OD450nm | | Mean OD | Corrected OD |
|--------------------------|---------|-------|------------|-----------------|
| | 1 | 2 | | |
| 10 | 2.419 | 2.42 | 2.42 | 2.358 |
| 5 | 1.734 | 1.763 | 1.749 | 1.687 |
| 2.5 | 1.018 | 1.022 | 1.02 | 0.958 |
| 1.25 | 0.511 | 0.52 | 0.516 | 0.454 |
| 0.63 | 0.272 | 0.275 | 0.274 | 0.212 |
| 0.32 | 0.149 | 0.149 | 0.149 | 0.087 |
| 0.16 | 0.102 | 0.102 | 0.102 | 0.04 |
| 0 | 0.061 | 0.062 | 0.062 | 0 |

Calculation of Results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Create a standard curve with standard concentration on the x-axis and OD values on the y-axis. Draw a best fit curve through the points and it can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor



Sensitivity

The minimum detectable dose of Rat HSPA4 is typically less than 0.1 ng/mL. The sensitivity of this assay, or Limit of Detection (LOD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by adding three standard deviations to the mean OD value of twenty zero standard replicates and calculating the corresponding concentration.

Specificity

This assay has high sensitivity and excellent specificity for detection of Rat HSPA4. No significant cross-reactivity or interference between Rat HSPA4 and analogues was observed. Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between Rat HSPA4 and all the analogues, therefore, cross reaction may still exist.

Precision

Mean coefficient of variation for Intra-Assay and Inter-Assay: 3 samples with low, middle and high level concentration were tested for repeat multiple times, respectively.

| Item | Intra-Assay | Inter-Assay |
|---------------|-------------|-------------|
| Sample number | 3 | 3 |
| Replicate | 9 | 18 |
| CV(%) | 5 | 8 |

Recovery

Three matrices listed below were spiked with certain level of Rat HSPA4, The recovery rates of Rat HSPA4 were calculated by comparing the measured value to the expected amount of Rat HSPA4 in samples.

| Matrix type | Recovery Range (%) | Average (%) |
|--------------------------|--------------------|-------------|
| Serum (n=5) | 82-95 | 88 |
| EDTA plasma (n=5) | 80-95 | 87 |
| Cell culture media (n=5) | 86-99 | 92 |

Linearity

Three types of Sample were spiked with appropriate concentrations of Rat HSPA4 and diluted into a series of concentration gradients, then the linearity of the assay was demonstrated by the percentage of comparing calculated concentrations and expected values.

| Dilution Factor | Recovery Range (%) | | |
|-----------------|--------------------|-------------------|--------------------------|
| | Serum (n=5) | EDTA plasma (n=5) | Cell culture media (n=5) |
| 1:2 | 95-104 | 80-92 | 82-93 |
| 1:4 | 82-93 | 86-102 | 96-105 |
| 1:8 | 81-96 | 87-103 | 87-98 |
| 1:16 | 83-94 | 85-101 | 85-97 |

Summary for Procedure



Add 50 μ L standard or sample to the wells, immediately add 50 μ L HRP conjugate working solution to each well. Incubate for 60 min at 37 $^{\circ}$ C



Aspirate and wash the plate for 3 times.



Add 100 μ L Substrate Reagent. Incubate for 15-20 min at 37 $^{\circ}$ C



Add 50 μ L Stop Solution



Read the plate at 450nm immediately. Calculation of the results

Troubleshooting

| Problem | Possible Causes | Subsequent Actions |
|------------------------------|--|---|
| Poor standard curve | Improper standard dilution | Ensure that standards are dissolved and diluted in the recommended manner |
| | Inaccurate pipetting | Periodically calibrate pipettes and check the pipette tips |
| | Evaporate the reaction solution | Seal the enzyme plate with plate sealer |
| | Incomplete plate washing | Adequate washing times and the amount of washing solution added |
| | Foreign matter in the bottom of wells | Clean the bottom of the plate before reading |
| Weak or no color development | Insufficient reaction of reagents | Ensure incubation time and incubate at the recommended temperature |
| | Inadequate reagent volumes | Check the pipette and Follow the steps strictly to operate |
| | Improper dilution | Check the reagent dilution process |
| | Inactivation of enzyme conjugate | Mix conjugate and substrate, check by color development |
| Low OD value | Incorrect setting of microplate reader | Check the wavelength of reader |
| | No stop solution added | Add appropriate amount of stop solution |
| | Waiting too long time to read | Read the plate in time |
| High background | Contaminated chromogenic solution | Replace chromogenic solution |
| | Coloring time is too long | Control the coloring time |
| | Wrong dilution of reaction reagent | Use the recommended dilution |
| | Inadequate washing of the plate | Adequate washing times and the amount of washing solution added |