INTRODUCTION

Heat Shock Protein 40 (HSP40) mRNA levels increase in various shrimp tissues during heat stress and infection (refs 1 & 2). HSP40 may provide a useful biomarker for assessment of shrimp health and development of disease resistant strains.

PRINCIPLE OF THE ASSAY

The assay uses polyclonal antibodies generated against recombinant Whiteleg Shrimp HSP40. Unconjugated antibodies are coated on wells of a microtiter plate and used for capture. Horseradish Peroxidase (HRP) conjugated antibodies are used for detection. Standards and diluted samples (100 µl) are incubated in the antibody coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 µl) is added and incubated for 45 minutes. If HSP40 molecules are present, they are sandwiched between the capture and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If HSP40 is present, a blue color develops. Color development is stopped by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of HSP40 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-HSP40 coated plate (12 x 8-well strips)
- HRP conjugate stock.
- HSP40 stock, 2 vials. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: SB50-1, 2 x 50 ml
- TMB: TMB11-1, 11 ml
- Stop Solution: SS11-1, 11 ml

Materials required but not provided:

- Pipettors and tips
- Distilled or deionized water
- Polypropylene tubes or 96-well polystyrene plates
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm
- Graphing software

STORAGE

Store the standard stock vials at -20°C. The remainder of the kit should be stored at 4°C and the microtiter plate should be kept in a sealed bag with desiccant. The kit will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature before use.
2. Reliable and reproducible results will be obtained when the assay is conducted with a complete understanding of the instructions and with adherence to good laboratory practice.
3. It is important that standards and samples be added to the ELISA plate quickly. If testing large numbers of samples, rather than pipetting standards and samples from individual tubes into the ELISA plate, we recommend the following: pipette an excess volume of standards and samples into wells of a blank polystyrene 96-well plate[1]. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 µl aliquots to the wells of the antibody-coated plate.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. Laboratory temperature will influence absorbance readings. The assay was calibrated using a shaking incubator set at 150 rpm and 25°C. Performing the assay at lower temperatures and mixing speeds may result in lower absorbance values.

WASH SOLUTION

The Wash Solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water. Unused wash buffer may be stored at 4°C for one week.

DILUENT

The diluent is formulated for measurement of HSP40 in shrimp tissue extracts. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. Reconstitute it with deionized water as detailed on the vial label, gently mix, and prepare the 30 ng/ml standard as described.
2. Label seven polypropylene tubes as 15, 7.5, 3.75, 1.88, 0.94, 0.47, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 30 ng/ml HSP40 standard into the tube labeled 15 ng/ml and mix. This provides the 15 ng/ml HSP40 standard.
4. Similarly prepare the 7.5 – 0.47 ng/ml standards by two-fold serial dilution.

Discard the stock after use.

[1] Standards and sample dilutions may also be prepared directly in a blank polystyrene plate.

HRP CONJUGATE

Approximately five minutes prior to use, prepare the working HRP conjugate by diluting the HRP stock with SB50-1 diluent as described on the stock vial label. Use 100 µl per well.

SAMPLES

We tested extracts from healthy Gulf Shrimp muscle. Extracts were prepared by homogenizing tissue with four volumes of TBS (150 mM NaCl, 10 mM Tris.HCl, pH 7.4) using either a Potter Elvehjem homogenizer or a Bullet Blender®. Supernatants obtained after microcentrifugation were tested after being diluted a further 10-fold or greater with diluent SB50-1. Because HSP40 levels vary with study conditions, optimal dilutions must be determined empirically.

PROCEDURE

1. Secure the desired number of 8-well strips in the cassette. Unused strips should be stored in a sealed bag with desiccant at 4°C.
2. Dispense 100 µl of standards and samples into the wells.
3. Incubate on a plate shaker at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5x with 1x Wash Solution using a plate washer (400 µl/well).
5. Dispense 100 µl of HRP conjugate into the wells.
6. Incubate on a plate shaker at 150 rpm and 25°C for 45-minutes.
7. Empty and wash the microtiter wells 5x with 1x Wash Solution using a plate washer (400 µl/well).
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 µl of TMB into each well.
10. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
11. After 20 minutes, stop the reaction by adding 100 µl of Stop Solution to each well.
12. Gently mix. It is important to make sure that all the blue color changes to yellow.
13. Read absorbance at 450 nm[2] with a plate reader within 5 minutes.

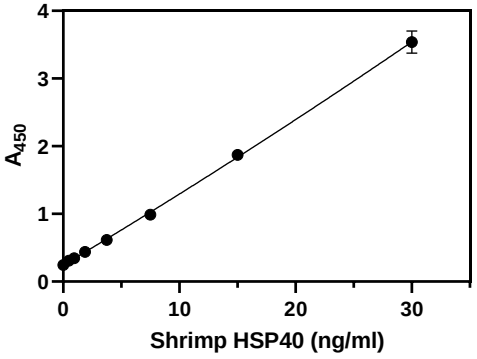
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the HSP40 concentration.
2. Fit the standard curve using graphing software. We suggest using a second order polynomial (quadratic)equation.
3. Derive the concentration of HSP40 in the samples.
4. Multiply the derived concentration by the dilution factor to determine the concentration in the sample.
5. If the absorbance values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve is shown below. This curve is for illustration only.

HSP40 (ng/ml)	A_{450}
30	3.537
15	1.870
7.5	0.989
3.75	0.614
1.88	0.439
0.94	0.344
0.47	0.307
0	0.245



[2] If absorbance of the high standard is ≥4 when measured at 450 nm, absorbance of all standards and samples should be read at 405 nm.

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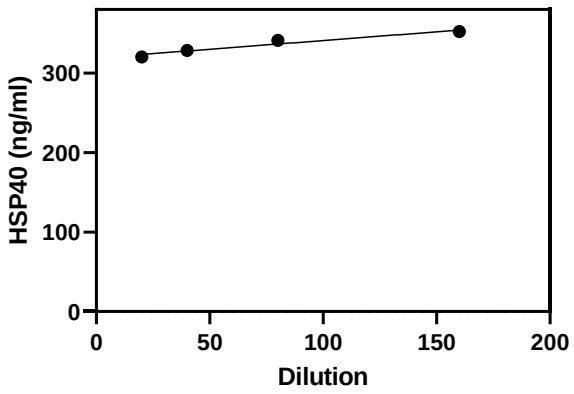
PERFORMANCE

Linearity: To assess the linearity of the assay, a muscle extracts from healthy Gulf Shrimp was serially diluted to produce values within the dynamic range of the assay.



REFERENCES

1. Chen T, et.al. Heat shock protein 40 (HSP40) in Pacific White Shrimp (Litopenaeus vannamei): molecular cloning, tissue distribution and ontogeny, response to temperature, acidity/alkalinity and salinity stresses, and potential role in ovarian development. Front. Physiol. 9:1784. doi: 10.3389/fphys.2018.01784
2. Ju-Ngam T, et.al. Functional and stress response analysis of heat shock proteins 40 and 90 of Giant River Prawn (Macrobrachium rosenbergii) under temperature and pathogenic bacterial exposure stimuli. Biomolecules 2021, 11, 1034. https://doi.org/10.3390/biom11071034

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