

INTRODUCTION

Heat Shock Protein 70 (HSP70) levels increase in Rainbow Trout heart, white muscle, red muscle, gill, and liver during heat stress (ref 1). Studies have also shown that it is increased in Salmon gill, muscle, and liver in response to elevated water temperatures (ref 2).

PRINCIPLE OF THE ASSAY

The assay uses a polyclonal antibody that recognizes Rainbow Trout and Salmon HSP70. Unconjugated antibodies are coated on wells of a microtiter plate and used for capture. Horseradish Peroxidase (HRP) conjugated antibody is used for detection. Standards and diluted samples (100 μ l) are incubated in the antibody coated microtiter wells for one hour. After washing the wells, HRP-conjugate (100 μ l) is added and incubated for 45 minutes. If HSP70 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 HSP70 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 HSP70 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-HSP70 coated plate (12 x 8-well strips)
- 2x HRP conjugate, 7 ml
- HSP70 stock, 2 vials. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: YD50-1, 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 vial 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 plate¹. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 μ l 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 HSP70 in trout and salmon tissue extracts. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. Reconstitute it with 200 μ l of deionized water, gently mix, and prepare the 30 ng/ml standard as described on the vial label.
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 HSP70 standard into the tube labeled 15 ng/ml and mix. This provides the 15 ng/ml HSP70 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

For each 8-well strip used in the assay, mix 0.5 ml of 2x HRP conjugate with 0.5 ml of YD50-1 diluent. Use 100 μ l per well.

SAMPLES

We tested extracts from salmon muscle and rainbow trout spleen. 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 YD50-1. Because HSP70 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 μ l of standards and samples into the wells.
3. Incubate on a plate shaker at 150 rpm and 25°C for one hour.
4. Empty and wash the microtiter wells 5x with 1x Wash Solution using a plate washer (400 μ l/well).
5. Dispense 100 μ l of 1x 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 μ l/well).
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 μ l 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 μ l 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 nm² with a plate reader within 5 minutes.

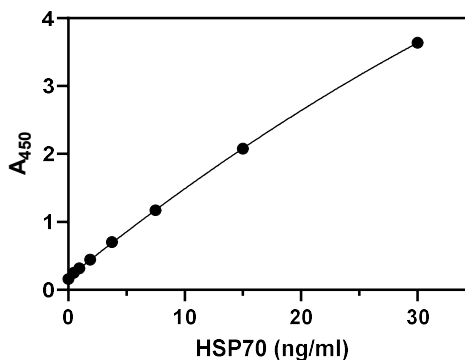
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the HSP70 concentration.
2. Fit the standard curve using graphing software. We suggest using a second order polynomial (quadratic) equation.
3. Derive the concentration of HSP70 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.

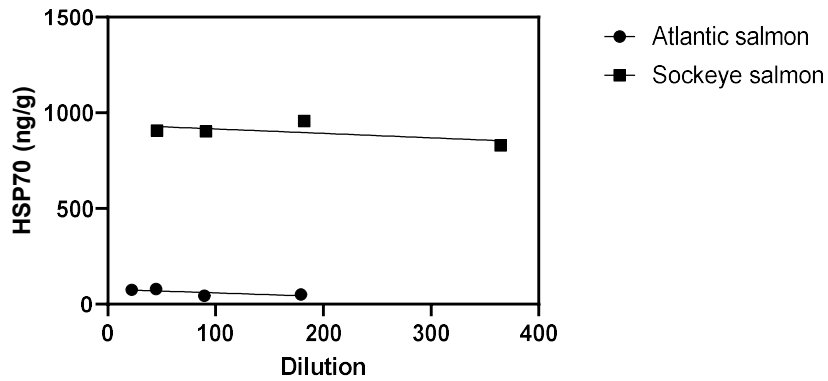
HSP70 (ng/ml)	A ₄₅₀
30	3.640
15	2.079
7.5	1.171
3.75	0.701
1.88	0.447
0.94	0.314
0.47	0.252
0	0.158



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.

PERFORMANCE

Linearity: To assess the linearity of the assay, muscle extracts from healthy Atlantic and Sockeye salmon were serially diluted to produce values within the dynamic range of the assay.



REFERENCES

1. Fowler SL, Hamilton D, and Currie S. A comparison of the heat shock response in juvenile and adult rainbow trout (*Oncorhynchus mykiss*) – implications for increased thermal sensitivity with age. *Can. J. Fish. Aquat. Sci.* 66:91-100 (2009)
2. Bowen L, et.al. Transcriptomic response to elevated water temperatures in adult migrating Yukon River Chinook salmon (*Oncorhynchus tshawytscha*). *Conserv Physiol* 8(1): coaa084; doi:10.1093/conphys/coaa084

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