INTRODUCTION

Inter-alpha-trypsin inhibitor heavy chain 4 (ITIH-4) is a serum protease inhibitor of ~105 kDa. It is a positive acute phase reactant; levels increase during inflammation, tissue injury, and bacterial infections. In studies at Life Diagnostics, we found levels of 21±9.6 μg/ml in serum from healthy beagles. In dogs with toxoplasmosis and liver disease, levels of 170 and 180 μg/ml were found.

PRINCIPLE OF THE ASSAY

The assay uses two peptide-specific dog ITIH-4 antibodies developed at Life Diagnostics. One is used as coating antibody. The other is conjugated to HRP and used for detection. Standards and diluted samples (100 μl) are incubated in anti-ITIH-4 coated microtiter wells for 45 minutes. After washing the wells, HRP-conjugate (100 μl) is added and incubated for 45 minutes. If ITIH-4 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 ITIH-4 is present, a blue color develops. Color development is stopped after 20-minutes by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of ITIH-4 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-ITIH-4 coated plate (12 x 8-well strips)
- Anti-ITIH-4 HRP Conjugate, 11 ml
- ITIH-4 stock, 1 vial. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- 10x Diluent: YD25-10, 25 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 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. 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.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
4. 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 (YD25-10) must be diluted prior to use. Mix one volume of YD25-10 with nine volumes of deionized water to obtain the working dilution buffer. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. It is comprised of dog ITIH-4 in a stabilizing matrix. Reconstitute it with deionized water as described on the vial label and gently mix. Prepare the 125 ng/ml standard as described on the label.
2. Label seven polypropylene tubes as 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 125 ng/ml ITIH-4 standard into the tube labeled 62.5 ng/ml and mix. This provides the 62.5 ng/ml ITIH-4 standard.
4. Similarly prepare the remaining standards by two-fold serial dilution.

IMPORTANT – If future use of the stock is intended, the sealed vial must be frozen at or below -20°C within 30 minutes of reconstitution.

HRP CONJUGATE

The HRP conjugate is provided ready to use. Warm to room temperature before use.

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

SAMPLES

The assay is intended for measurement of ITIH-4 in dog serum or plasma. A dilution of 5,000-fold worked well for most samples, but optimal dilutions should be determined by the end user. Ten-fold diluted YD25-10 must be used for sample dilution. A 5,000-fold dilution can be obtained as follows.

1. Dispense 198 µl and 294 µl of diluent into separate microcentrifuge tubes, or wells of a 96-well polystyrene plate.
2. Dilute 2.0 µl of serum or plasma into the tube containing 198 µl of diluent and mix. This provides a 100-fold dilution.
3. Dilute 6.0 µl of the 100-fold diluted sample into the tube containing 294 µl of diluent and mix. This provides a 5,000-fold dilution.

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 appropriate wells. We recommend that standards and samples be tested in duplicate.
3. Incubate on a plate shaker at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5 times 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 5 times with 1x Wash Solution using a plate washer (400 µl/well).
8. If necessary, 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.

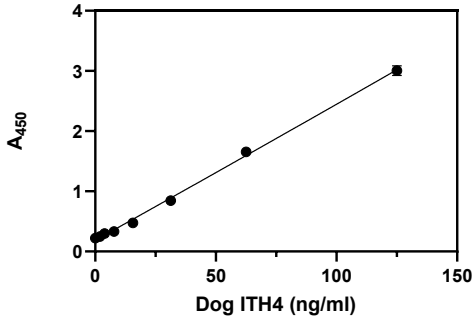
RESULTS

1. Using curve fitting software, graph the absorbance values of the standards on the Y-axis versus ITIH-4 concentration on the X-axis.
2. Fit the curve to a second order polynomial (quadratic) equation and derive the concentration of ITIH-4 in the diluted samples.
3. Multiply derived values by the dilution factor(s) to determine ITIH-4 concentration in the original sample.
4. If the absorbance values of diluted samples fall outside the standard curve, samples should be further diluted and re-tested.

TYPICAL STANDARD CURVE

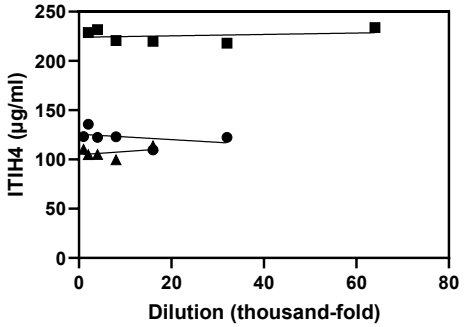
A typical standard curve is shown below. This curve is for illustration only.

ITIH-4 (ng/ml)	A_{450}
125	3.007
62.5	1.654
31.25	0.845
15.63	0.474
7.81	0.333
3.91	0.298
1.95	0.250
0	0.223



PERFORMANCE

Linearity: To assess the linearity of the assay, serum samples with ITIH-4 concentrations of 107, 123 and 226 µg/ml were diluted with to give values within range of the assay.



Rev 112625

For technical assistance please email us: techsupport@lifediagnostics.com

[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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