

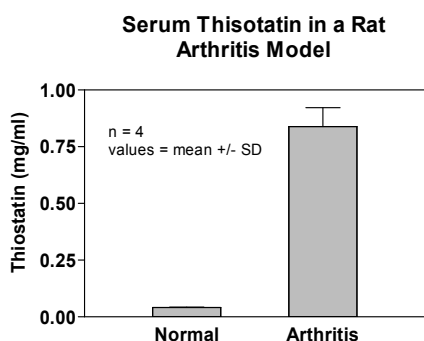
RAT THIOSTATIN ELISA TEST KIT

Life Diagnostics, Inc., Catalog Number: 3000-2

Enzyme Immunoassay for the Quantitative Determination of Rat Thiostatin in Serum or Plasma

INTRODUCTION

Thiostatin is glycoprotein with a molecular weight of 56,000^{1,2}. It is a cysteine protease inhibitor that is also known as major acute phase protein (α 1-MAP)². In response to inflammation serum levels of thiostatin increase as much as 20-fold^{1,3}. As shown in the figure below, studies at Life Diagnostics indicate a significant increase in serum thiostatin in a rat arthritis model. Thiostatin thus serves as an excellent marker of inflammation and disease in rats.



PRINCIPLE OF THE TEST

The rat thiostatin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat thiostatin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat thiostatin antibodies for detection. The test sample is diluted and incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed and HRP conjugate is added and incubated for 45 minutes. This results in thiostatin molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-labeled antibodies and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of thiostatin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-rat thiostatin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized), containing 2 μ g/ml rat thiostatin when reconstituted as detailed on the vial label
- 10x Diluent (25 ml)
- 20x Wash Solution (50 ml)
- TMB Reagent (One-Step) 11 ml

- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate reader with an optical density range of 0-4 at 450 nm
- Graph paper (PC graphing software is optional).

STORAGE OF TEST KIT AND INSTRUMENTATION

The unopened kit should be stored at 2-8°C and the microtiter plate should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase if the components are stored as described above.

GENERAL INSTRUCTIONS

All reagents should be allowed to reach room temperature (18-25°C) before use.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

WASH SOLUTION PREPARATION

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.

STANDARD PREPARATION

1. The rat thiostatin standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized water indicated on the vial label and mix gently until dissolved to obtain a 2 μ g/ml rat thiostatin stock (*the reconstituted standard remains stable for at least 1 day at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended*).
2. Label 8 polypropylene or glass tubes as 75, 37.5, 18.75, 9.38, 4.67, 2.34, 1.17 and 0 ng/ml.
3. Dispense 962.5 μ l of diluent into the tube labeled 75 ng/ml and 300 μ l of diluent into the remaining tubes.
4. Pipette 37.5 μ l of the 2 μ g/ml thiostatin standard into the tube labeled 75 ng/ml and mix. This provides the working 75 ng/ml thiostatin standard.
5. Prepare a 37.5 ng/ml standard by diluting and mixing 300 μ l of the 75 ng/ml standard with 300 μ l of diluent in the tube labeled 37.5 ng/ml. Similarly prepare the 18.75, 9.38, 4.67, 2.34 and 1.17 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: We found that thiostatin is generally present in normal rat serum at a concentration of ~50 µg/ml and may increase to ~1 mg/ml as a result of inflammation. In order to obtain values within the range of the standard curve, we suggest that samples initially be diluted 10,000 fold using the following procedure for each sample to be tested:

1. Dispense 495 µl and 297 µl of 1x diluent into separate tubes.
2. Pipette and mix 5 µl of the serum/plasma sample into the tube containing 495 µl of diluent. This provides a 100 fold diluted sample.
3. Mix 3 µl of the 100 fold diluted sample with the 297 µl of diluent in the second tube. This provides a 10,000 fold dilution of the sample.
4. Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 µl of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
4. Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may be performed using either a plate washer (400 µl/well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 µl of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
9. Wash as detailed in 4 to 6 above.
10. Dispense 100 µl of TMB Reagent into each well.
11. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
12. Stop the reaction by adding 100 µl of Stop Solution to each well.
13. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
14. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

CALCULATION OF RESULTS

1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of thiostatin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of thiostatin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.

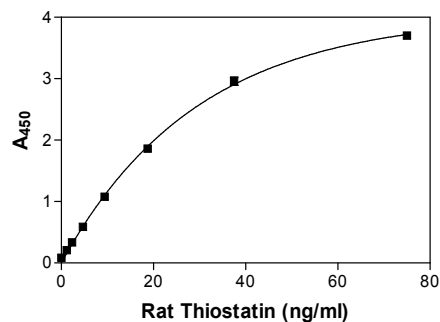
6. If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 10,000 samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450 nm on the Y axis against thiostatin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Thiostatin (ng/ml)	Absorbance (450 nm)
75	3.702
37.5	2.959
18.75	1.863
9.38	1.077
4.67	0.588
2.34	0.336
1.17	0.210
0	0.085

Representative Rat Thiostatin Standard Curve



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions detailed above.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. J Urban et.al., A rat serum glycoprotein whose synthesis rate increases greatly during inflammation. J Biol Chem. 254:10565-10568 (1979)
2. KP Anderson and EC Heath. The relationship between rat major acute phase protein and the kininogens. J Biol Chem. 260:12065-12071 (1985)
3. I Miller et.al., Proteins of rat serum: III. Gender-related differences in protein concentration under baseline conditions and upon experimental inflammation as evaluated by two-dimensional electrophoresis. Electrophoresis 20:836-845 (1999)