

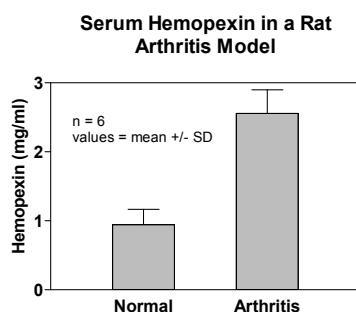
RAT HEMOPEXIN ELISA TEST KIT

Life Diagnostics, Inc., Catalog Number: 2910-2

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

INTRODUCTION

Hemopexin is an acute phase protein that is elevated 2-3 fold in rat serum and plasma as a result of inflammation^{1,2}. As illustrated in the figure below, studies at Life Diagnostics Inc. indicate that hemopexin is elevated approximately 2.5 fold in serum of arthritic rats. Hemopexin provides a convenient marker of inflammation and tissue injury in the rat.



PRINCIPLE OF THE TEST

The rat hemopexin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat hemopexin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat hemopexin 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 hemopexin 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 hemopexin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-rat hemopexin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized) containing 2 µg/ml rat hemopexin
- 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 with an approximate mixing speed of 150 rpm
- A microtiter plate reader at 450 nm wavelength, with a bandwidth of 10 nm or less and an optical density range of 0-4.
- 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 provided that the components are stored as described above.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~25,000 fold with 1x diluent in order to obtain values within the standard range.

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. Add the volume of distilled or de-ionized water indicated on the lyophilized rat hemopexin standard vial label to the standard vial and mix gently until dissolved. This provides a 2 µg/ml stock (**the reconstituted standard should be aliquoted and frozen at -20°C after reconstitution if future use is intended**).
2. Label 8 polypropylene microcentrifuge tubes as 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml
3. Dispense 875 µl of 1x diluent into the tube labeled 250 ng/ml and 300 µl of diluent into the remaining tubes.

- Pipette 125 μl of the 2 $\mu\text{g}/\text{ml}$ hemopexin standard into the tube labeled 250 ng/ml and mix. This provides the working 250 ng/ml hemopexin standard.
- Prepare a 125 ng/ml standard by diluting and mixing 300 μl of the 250 ng/ml standard with 300 μl of diluent in the tube labeled 125 ng/ml. Similarly prepare the 62.5, 31.25, 15.6, 7.8, and 3.9 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Hemopexin is generally present in rat serum at concentrations ranging from 1-3 mg/ml. In order to obtain values within the range of the standard curve samples should be diluted 25,000 fold. We suggest the following procedure for each sample to be tested:

- Dispense 497.5 μl and 248 μl of 1x diluent into separate polypropylene tubes.
- Pipette and mix 2.5 μl of the serum/plasma sample into the tube containing 497.5 μl of diluent. This provides a 200 fold diluted sample.
- Mix 2.0 μl of the 200 fold diluted sample with the 248 μl of diluent in the second tube. This provides a 25,000 fold dilution of the sample.
- Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

- Secure the desired number of coated wells in the holder.
- Dispense 100 μl of standards and samples into the wells (we recommend that samples be tested in duplicate).
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- Remove the incubation mixture by flicking plate contents into an appropriate Bio-waste container or using a plate washer.
- Wash and empty the microtiter wells 5 times with 1x wash solution. This may be performed using either a plate washer (400 $\mu\text{l}/\text{well}$) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
- Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
- Add 100 μl of enzyme conjugate reagent into each well.
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- Wash as detailed in 4 to 5 above.
- Strike the wells sharply onto absorbent paper or paper towels to remove residual water droplets.
- Dispense 100 μl of TMB Reagent into each well.
- Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
- Stop the reaction by adding 100 μl of Stop Solution to each well.
- Gently mix. *It is important to make sure that all the blue color changes to yellow.*
- Read the optical density at 450 nm with a microtiter plate reader *within 15 minutes.*

CALCULATION OF RESULTS

- Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.

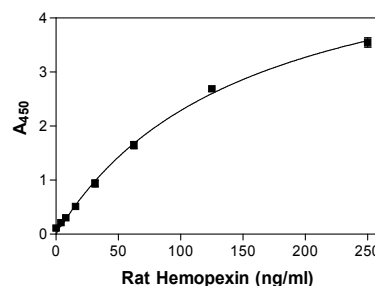
- Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
- Using the mean absorbance value for each sample, determine the corresponding concentration of hemopexin in ng/ml from the standard curve.
- Multiply the derived concentrations by the dilution factor to determine the actual concentration of hemopexin in the serum/plasma sample.
- PC graphing software may be used for the above steps.
- If the OD_{450} values of samples fall outside the standard curve when tested at a dilution of 25,000, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against hemopexin 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.

Hemopexin (ng/ml)	Absorbance (450 nm)
250	3.546
125	2.687
62.5	1.649
31.25	0.938
15.6	0.512
7.8	0.301
3.9	0.210
0	0.112

Typical Rat Hemopexin Standard Curve



LIMITATIONS OF THE PROCEDURE

- 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.
- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

1. 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)
2. S Marainkovic et.al. IL-6 modulates the synthesis of a specific set of acute phase plasma proteins in vivo. *J Immunol*. 142:808-812 (1989)