RAT ALPHA-2-MACROGLOBULIN ELISA
Life Diagnostics, Inc., Catalog Number: MAC-2

RAT ALPHA-2-MACROGLOBULIN ELISA

INTRODUCTION
Alpha-2-macroglobulin is an acute phase protein that is elevated in serum or plasma as a result of injury, infection or disease. It functions as a broad range inhibitor of proteases such as trypsin and is believed to provide a protective role during the acute phase response. Studies at Life Diagnostics, Inc. indicate that alpha-2-macroglobulin levels increase 75-150 fold during chronic disease in rats. Measurement of alpha-2-macroglobulin provides an excellent marker of inflammation and disease.

PRINCIPLE OF THE TEST
The rat alpha-2-macroglobulin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-rat alpha-2-macroglobulin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat alpha-2-macroglobulin antibodies for detection. The test sample is first mixed with trypsin and incubated for one hour at room temperature. The trypsin reaction is then stopped by addition of trypsin inhibitor. Trypsin treatment normalizes reactivity of the alpha-2-macroglobulin isoforms in non-acute phase and acute phase serum. The sample is then appropriately 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 alpha-2-macroglobulin 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 alpha-2-macroglobulin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS
Materials provided with the kit:
- Anti-rat alpha-2-macroglobulin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Trypsin (lyophilized, 1ml)
- Trypsin inhibitor (lyophilized, 1 ml)
- Enzyme Conjugate Reagent, 11 ml
- Alpha-2-macroglobulin stock (lyophilized)
- 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 tubes
- Vortex mixer
- Absorbent paper or paper towels
- Plate shaker with an approximate mixing speed of 150 rpm
- Plate washer
- 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
The 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.

GENERAL INSTRUCTIONS
1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Optimum results are achieved if, at each step, reagents are pipetted into the wells of the microtiter plate within 5 minutes.

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.

TRYPSIN / TRYPsin INHIBITOR PREPARATION
Trypsin and trypsin inhibitor are provided as lyophilized stocks. Prior to use reconstitute each vial with 1 ml of distilled or deionized water. Store the reconstituted stock at -20°C.

STANDARD PREPARATION
1. The rat alpha-2-macroglobulin 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 (the reconstituted standard should be aliquoted and frozen at or below -20°C if further use is intended).
2. Label 7 polypropylene or glass tubes as 500, 250, 125, 62.5, 31.25, 15.6, and 7.8 ng/ml.
3. Prepare the 500 ng/ml standard as indicated on the stock vial label.
4. Dispense 250 µl of diluent into the tubes labeled 250, 125, 62.5, 31.25, 15.6, and 7.8 ng/ml.
5. Prepare a 250 ng/ml standard by diluting and mixing 250 µl of the 500 ng/ml standard with 250 µl of diluent in the tube labeled 250 ng/ml. Similarly prepare the 125, 62.5, 31.25, 15.6, and 7.8 ng/ml standards by serial dilution.

SAMPLE PREPARATION
A. Trypsin Treatment
1. For each sample to be tested, pipet 18 µl of trypsin into a polypropylene microcentrifuge tube.
2. Add 2 μl of the serum or plasma test sample and mix.
3. Incubate for 1 hour at room temperature.
4. Add 20 μl of Trypsin Inhibitor and mix.
5. At this point the samples may be tested as described below or they may be frozen at or below -20°C for future use.
6. Please note that as a result of the trypsin/trypsin inhibitor treatment the samples are diluted 20-fold.

B. Dilution
Studies at Life Diagnostics, Inc., indicate that alpha-2-macroglobulin is present in normal rat serum at concentrations of approximately 25 μg/ml and that levels can increase to approximately 5 mg/ml in acute phase serum. In order to ensure that values of test samples fall within the range of the standard curve we suggest that each sample be tested at dilutions of 1000 and 50,000. These dilutions may be achieved as follows:

1000 Fold Dilution
1. Pipette and mix 8 μl of each trypsin treated serum/plasma sample into a tube containing 392 μl of diluent. This provides a 1000 fold diluted sample relative to the original serum/plasma sample.

50,000 Fold Dilution
1. Pipette and mix 10 μl of the 1000-fold diluted serum/plasma sample into a tube containing 490 μl of diluent. This provides a 50,000 fold diluted sample.
2. 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. Empty and wash the microtiter wells 5 times with 400 μl of 1x wash solution using a plate washer. The entire wash procedure should be performed as quickly as possible.
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
6. Add 100 μl of enzyme conjugate reagent into each well.
7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
8. Wash as detailed in 4-5 above.
9. Dispense 100 μl of TMB Reagent into each well.
10. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
11. 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 the optical density at 450 nm with a microtiter plate reader within 5 minutes.

CALCULATION OF RESULTS
1. Calculate the average absorbance values (A450) 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 alpha-2-macroglobulin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of alpha-2-macroglobulin in the serum/plasma sample.
5. If available, PC graphing software may be used for the above steps.
6. If the OD450 values of samples fall outside the limits of the standard curve 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 alpha-2-macroglobulin 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.

<table>
<thead>
<tr>
<th>Alpha-2-macroglobulin (ng/ml)</th>
<th>A450</th>
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<tbody>
<tr>
<td>500</td>
<td>3.296</td>
</tr>
<tr>
<td>250</td>
<td>2.051</td>
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<tr>
<td>125</td>
<td>1.149</td>
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<tr>
<td>62.5</td>
<td>0.714</td>
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<tr>
<td>31.25</td>
<td>0.414</td>
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<tr>
<td>15.6</td>
<td>0.256</td>
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<tr>
<td>7.8</td>
<td>0.186</td>
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</tbody>
</table>

LIMITATIONS OF THE PROCEDURE
1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the instructions and with adherence to good laboratory practice. Please read the entire kit insert before starting the assay.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

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