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

INTRODUCTION

Alpha-2-macroglobulin (A2M) is an acute phase protein that is elevated in serum or plasma because of injury, infection or disease.[1,2] It functions as an inhibitor of proteases such as trypsin and is believed to provide a protective role during the acute phase response. At Life Diagnostics, Inc. we found that A2M levels increase up to 150-fold during chronic disease in rats.

PRINCIPLE OF THE ASSAY

The assay uses affinity purified rat A2M antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated rat A2M antibodies for detection. Samples are 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 A2M isoforms in non-acute phase and acute phase serum. Standards and diluted samples are then incubated in the microtiter wells for 45 minutes. The wells are subsequently washed. HRP conjugate is added and incubated for 45 minutes. This results in A2M molecules being sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If A2M is present a blue color develops. Color development is stopped by the addition of Stop solution, changing the color to yellow, and absorbance is measured at 450 nm. The concentration of A2M is proportional to absorbance and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:
- A2M antibody coated 96-well plate (12 x 8-well strips)
- HRP Conjugate, 11 ml
- A2M stock (lyophilized)
- Trypsin (lyophilized)
- Trypsin inhibitor (lyophilized)
- 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 or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm.
- Curve fitting software

STORAGE

The unused kit should be stored at 4°C and the microtiter plate should be kept in a sealed bag with desiccant. Kits 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 carried out with a complete understanding of the instructions and with adherence to good laboratory practice.
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. Our ELISA kits are calibrated using shaking incubators set at 150 rpm and 25°C. Performance of the assay at lower temperatures will result in lower absorbance values.

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 volume of the 10x stock with nine 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. Reconstitute each vial with 1 ml of distilled or deionized water. Store the reconstituted stock at -20°C.

STANDARD PREPARATION

1. The A2M stock is provided lyophilized. Add the volume of distilled or deionized water indicated on the vial label and mix *(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 mixing 250 µl of the 500 ng/ml standard with 250 µl of diluent in the tube labeled 250 ng/ml.
6. Similarly prepare the remaining standards by two-fold serial dilution.

SAMPLE PREPARATION

A. Trypsin Treatment

1. For each sample, pipet 18 µl of trypsin into a polypropylene microcentrifuge tube.
2. Add 2.0 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 because of the trypsin/trypsin inhibitor treatment the samples are diluted 20-fold.

B. Dilution

Studies at Life Diagnostics, Inc., indicate that A2M 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. 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. Mix 8.0 μl of each trypsin treated serum/plasma sample into a tube containing 392 μl of diluent. This provides a 1000-fold dilution.

50,000-Fold Dilution

1. Mix 10 μl of the 1000-fold diluted sample into a tube containing 490 μl of diluent. This provides a 50,000-fold diluted sample.

ASSAY PROCEDURE

1. Secure the desired number of 8-well strips in the holder. Unused strips should be stored in the re-sealed bag with desiccant at 4°C for future use.
2. Dispense 100 μl of standards and samples into the wells (we recommend that standards and samples be run in duplicate).
3. Incubate on an orbital micro-plate shaker at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5x with 1x wash solution using a plate washer (400 μl/well).
5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
6. Add 100 μl of HRP-conjugate into each well.
7. Incubate on a plate shaker at 150 rpm and 25°C for 45 minutes.
8. Wash as detailed above.
9. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
10. Dispense 100 μl of TMB into each well.
11. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
12. After 20-minutes, 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 absorbance at 450 nm with a plate reader within 5 minutes.

CALCULATION OF RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus concentration.
2. Fit the standard curve to an appropriate model and derive the concentration of the samples (we recommend using a single site, total and nonspecific binding model).
3. Multiply the derived concentration by the dilution factor to determine the actual concentration in the serum sample.
4. If the A_{450} values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with absorbance at 450 nm on the Y-axis against A2M concentrations on the X-axis is shown below. This curve is for illustration only.

A2M (ng/ml)	A_{450}
500	3.296
250	2.051
125	1.149
62.5	0.714
31.25	0.414
15.6	0.256
7.8	0.186
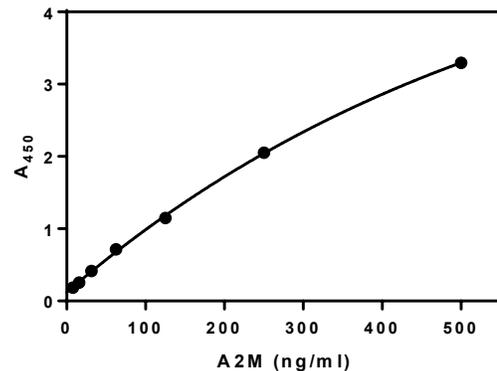
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1. KL Thomsen, et al. Regulation of urea synthesis during the acute phase response in rats. Am J Physiol Gastrointest Liver Physiol. 304: G680–G686 (2013)
2. KJ Wedekind, et. al. Beneficial effects of natural eggshell membrane (NEM) on multiple indices of arthritis in collagen-induced arthritic rats, Modern Rheumatology, 27:5, 838-848 (2016) (https://doi.org/10.1080/14397595.2016.1259729)

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