Rabbit Alpha 2-Macroglobulin ELISA Quantitation Kit

Manual

Catalog number: 40-374-130002

Immunoperoxidase Assay for Determination of Alpha 2-Macroglobulin in Rabbit Sera.
This kit is for research use only, and is not for use in diagnostic procedures.

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INTENDED USE

The ALPHA 2-MACROGLOBULIN test kits are a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring ALPHA 2-MACROGLOBULIN in the biological samples of Rabbits.

INTRODUCTION

Alpha 2-Macroglobulin (A2M) is a major protease inhibitor in serum and an acute phase protein which increases significantly in concentration in the Rabbit as a result of inflammation. The major pathophysiological role for Rabbit alpha 2-macroglobulin has yet to be conclusively defined. This kit is specific to the alpha 2-macroglobulin and will not cross react with the closely related alpha 1-macroglobulin.

PRINCIPLE OF THE TEST

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the Alpha 2-Macroglobulin present in samples react with the anti-A2M. antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti-A2M. antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound A2M. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of Alpha 2-Macroglobulin in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of Alpha 2-Macroglobulin in the test sample. The quantity of Alpha 2-Macroglobulin in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for dilution.

ANTI-A2M ANTIBODIES BOUND TO SOLID PHASE
CONTROL AND PATIENT SERUM SAMPLES ADDED
A2M * ANTI-A2M COMPLEXES FORMED
UNBOUND SERUM PROTEINS REMOVED
ANTI-A2M-HRP CONJUGATE ADDED
ANTI-A2M-HRP * A2M * ANTI-A2M COMPLEXES FORMED
UNBOUND ANTI-A2M-HRP REMOVED
CHROMOGENIC SUBSTRATE ADDED
DETERMINE BOUND ENZYMATIC ACTIVITY

FIGURE 1.
REAGENTS

(Quantities sufficient for 96 determinations)

1. DILUENT CONCENTRATE
One bottle containing 50 mL of a 5X concentrated phosphate buffered saline (PBS) solution containing bovine serum albumin, 0.25% Tween, and 0.1% Proclin 300 as a preservative.

2. WASH SOLUTION CONCENTRATE
One bottle containing 50 mL of a 20X concentrated phosphate buffered saline (PBS) solution containing 0.5% Tween.

3. ENZYME-ANTIBODY CONJUGATE 100X
One vial containing 200 μL of affinity purified anti-Pig HATOGLOBIN antibody conjugated with horseradish peroxidase in a stabilizing buffer.

4. CHROMOGEN-SUBSTRATE SOLUTION
One vial containing 12 mL of 3,3',5,5'-tetramethylenbenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

5. STOP SOLUTION
One vial containing 12 mL 0.3 M sulfuric acid.

WARNING: Avoid contact with skin.

6. ANTI-RABBIT ALPHA 2-MACROGLOBULIN ELISA MICRO PLATE
Twelve removable eight (8) well micro well strips in well holder frame. Each well is coated with affinity purified anti-Rabbit Alpha 2-Macroglobulin.

7. Rabbit ALPHA 2-MACROGLOBULIN CALIBRATOR
One vial containing a lyophilized Rabbit Alpha 2 Macroglobulin calibrator.

FOR IN VITRO USE ONLY

REAGENT PREPARATION

1. DILUENT CONCENTRATE
The Diluent Solution supplied is a 5X Concentrate and must be diluted 1:5 with distilled or deionized water.

2. WASH SOLUTION CONCENTRATE
The Wash Solution supplied is a 20X Concentrate and must be diluted 1:20 with distilled or deionized water. Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.

3. ENZYME-ANTIBODY CONJUGATE
The required amount of working conjugate solution for each microtitre plate is prepared by adding 100 μL Enzyme-Antibody Conjugate to 10 mL of Diluent. Mix uniformly, but gently. Avoid foaming.

4. CHROMOGEN-SUBSTRATE SOLUTION
Ready to use as supplied.

5. STOP SOLUTION
Ready to use as supplied.
6. ANTI-RABBIT ALPHA 2-MACROGLOBULIN ELISA MICRO PLATE
Ready to use as supplied.

7. RABBIT ALPHA 2-MACROGLOBULIN CALIBRATOR
Add 2.0 ml of distilled or de-ionized water to the Rabbit Alpha 2 Macroglobulin Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 110 μg/ml (the reconstituted calibrator should be aliquoted and frozen if future use is intended). Rabbit Alpha 2 Macroglobulin standards need to be prepared immediately prior to use (see the following chart). Mix well between each step. Avoid foaming.

<table>
<thead>
<tr>
<th>Standard</th>
<th>ng/mL</th>
<th>Volume added to 1x Diluent</th>
<th>Volume of 1x Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>400</td>
<td>2 µl Pig Hp Calibrator</td>
<td>548 µl</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>250 µl standard 1</td>
<td>250 µl</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>250 µl standard 2</td>
<td>250 µl</td>
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<tr>
<td>4</td>
<td>50</td>
<td>250 µl standard 3</td>
<td>250 µl</td>
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<tr>
<td>5</td>
<td>25</td>
<td>250 µl standard 4</td>
<td>250 µl</td>
</tr>
<tr>
<td>6</td>
<td>12.5</td>
<td>250 µl standard 5</td>
<td>250 µl</td>
</tr>
<tr>
<td>7</td>
<td>6.25</td>
<td>250 µl standard 6</td>
<td>250 µl</td>
</tr>
</tbody>
</table>

**STORAGE AND STABILITY**

The expiration date for the package is stated on the box label.

1. DILUENT
The 5X Diluent Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4-8°C.

2. WASH SOLUTION
The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions can be stored at room temperature (16-25°C) or at 4-8°C.

3. ENZYME-ANTIBODY CONJUGATE
Undiluted horseradish peroxidase anti-HX conjugate should be stored at 4-8°C and diluted immediately prior to use. The working conjugate solution is stable for one day.

4. CHROMOGEN-SUBSTRATE SOLUTION
The Substrate Solution should be stored at 4-8°C and is stable until the expiration date.

5. STOP SOLUTION
The Stop Solution should be stored at 4-8°C and is stable until the expiration date.

6. ANTI-RABBIT ALPHA 2-MACROGLOBULIN ELISA MICRO PLATE
Anti-Rabbit A2M coated wells are stable until the expiration date, and should be stored at 4-8°C in the sealed foil pouch with desiccant pack.

7. RABBIT ALPHA 2-MACROGLOBULIN STANDARDS
The lyophilized Rabbit Alpha 2 Macroglobulin Calibrator should be stored at 4C or frozen until reconstituted. The reconstituted calibrator should be aliquoted and stored frozen (avoid multiple freeze-thaw cycles). The working standard solutions should be prepared immediately prior to use and are stable for 1 day.
INDICATIONS OF INSTABILITY

If the test is performing correctly, the results observed with the standard solutions should be within 20% of the expected values.

SPECIMEN COLLECTION AND PREPARATION

Blood should be collected by venipuncture and the serum separated from the cells, after clot formation, by centrifugation. Specimens may be shipped at room temperature and then stored refrigerated at 2-8°C if testing is to take place within one week after collection. If testing is to take place later than one week, specimens should be stored at -20°C. Avoid repeated freeze-thaw cycles.

1. Precautions

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds.

2. Additives and Preservatives

No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

3. Known interfering substances

Azide and thimerosal at concentrations higher than 0.1% inhibits the enzyme reaction.

MATERIAL PROVIDED

See "REAGENTS"

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipette (2 μL to 200 μL) for making and dispensing dilutions
- Test tubes
- Microtitre washer/aspirator
- Distilled or Deionized H₂O
- Microtitre Plate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Vortex mixer

ASSAY PROCEDURE

DILUTION OF SERUM SAMPLES

The assay for quantification of Alpha 2-Macroglobulin in serum requires that each test sample be diluted before use. A 1:50,000 dilution is appropriate for most serum or plasma samples. For absolute quantification,
samples that yield results outside the range of the standard curve, a lesser or greater dilution might be required.

1. To prepare a 1:50,000 dilution of sample, transfer 2 μL of sample to 998 μL of diluent. This gives you a 1:500 dilution. Next, dilute the 1:500 samples by transferring 5 μL to 495 μL of diluent. You now have a 1:50,000 dilution of your sample. Mix thoroughly at each stage.

PROCEDURE

Bring all reagents to room temperature before use.

1. Add 100 μL of Diluent to each of the wells in 1A & 2A. These will serve for an evaluation of the background associated with the assay.

2. Pipette 100 μL of
   - Standard 1 (400 ng/ml) into wells 1B & 2B
   - Standard 2 (200 ng/ml) into wells 1C & 2C
   - Standard 3 (100 ng/ml) into wells 1D & 2D
   - Standard 4 (50 ng/ml) into wells 1E & 2E
   - Standard 5 (25 ng/ml) into wells 1F & 2F
   - Standard 6 (12.5 ng/ml) into wells 1G & 2G
   - Standard 7 (6.25 ng/ml) into wells 1H & 2H

3. Pipette 100 μL of serum sample (test sample 1) into wells 3A & 4A. The next sample goes in wells 3B & 4B, the next in 3C & 4C and so on.

4. Incubate the micro titer plate at 22°C (room temperature) for thirty (30 ± 2) minutes. Keep plate level during incubation.

5. Following incubation, aspirate the contents of the wells.

6. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually; completely fill wells with wash buffer, invert the plate and pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.

7. Pipette 100 μL of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (room temperature) for thirty (30 ± 2) minutes.

8. Wash and blot the wells as described in Step 5/6.

9. Pipette 100 μL of TMB Substrate Solution into each well.

10. Incubate at room temperature for precisely ten (10) minutes.

11. After ten (10) minutes, add 100 μL of Stop Solution to each well.

12. Determine the absorbance (450 nm) of the contents of each well. Calibrate the plate reader to air.

STABILITY OF THE FINAL REACTION MIXTURE

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates that the measurement be made as soon as possible.
RESULTS

1. Subtract the average background value from the test values for each sample.

2. Using the results observed for the standards construct a Standard Curve. The appropriate curve fit is that of a four-parameter logistics curve. A second order polynomial (quadratic) or other curve fits may also be used.

3. Interpolate test sample values from standard curve. Correct for sera dilution factor to arrive at the Alpha 2-Macroglobulin concentration in original sample.

QUALITY CONTROL

In accord with good laboratory practice, the Assays for specific A2M require meticulous quality control. Each laboratory should use routine quality control procedures to establish inter- and intra-assay precision and performance characteristics.

LIMITATION OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice.

2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or deionized water, and accuracy of reagent and sample pipettings.

TROUBLESHOOTING

The following are some common problems encountered with the use of ELISA kits, and some of the causes of these problems.

1. **Problem: Low absorbance**
   - Incorrect dilutions or pipetting errors.
   - Improper incubation times
   - Improper mixing of the TMB substrate. Each component is mixed in equal parts.
   - Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB, 490 nm for OPD, or 405 nm for ABTS.
   - Kit materials or reagents are contaminated or expired.
   - Incorrect reagents used.

2. **Problem: High Absorbance**
   - Cross contamination from other samples or positive control.
   - Incorrect dilutions or pipetting errors.
   - Improper washing.
   - Wrong filter on microtiter reader.
   - Contaminated buffers or enzyme substrate.
   - Improper incubation times.
   - Kit materials or reagents are contaminated or expired.
3. **Problem: Poor Duplicates**
   - Poor mixing of specimens.
   - Incorrect dilutions or pipetting errors.
   - Technical error.
   - Inconsistency in following ELISA protocol.
   - Inefficient washing.

4. **Problem: All wells are positive**
   - Contaminated buffers or enzyme substrate.
   - Incorrect dilutions or pipetting errors.
   - Kit materials or reagents are contaminated or expired.
   - Inefficient washing.

5. **Problem: All wells are negative**
   - Procedure not followed correctly.
   - Contaminated buffers or enzyme substrate.
   - Contaminated conjugate.
   - Kit materials or reagents are contaminated or expired.

The above information is believe to be correct but does not purport to be all-inclusive and is intended to be used only as a guide. GenWay Biotech, Inc. shall not be liable or responsible in any way for use of either this information or the material supplied. Disposal of hazardous material may be subject to federal, state or local laws or regulations.