Manual

α₂-Macroglobulin ELISA Kit

For the in vitro determination of α₂-Macroglobulin in urine, serum and plasma

Valid from 12.11.2012

REF K 6610 Σ 96 +2°C +8°C IVD CE
1. INTENDED USE

The Immundiagnostik Assay is intended for the quantitative determination of α-2-Macroglobulin in urine, serum and plasma. For in vitro diagnostic use only.

2. SUMMARY AND EXPLANATION OF THE TEST

Alpha-2-Macroglobulin is one of the biggest plasma proteins with a molecular weight of 650-900kDa, dependent on the degree of glycosylation. It consists of 4 subunits. Alpha-2-Macroglobulin functions as protease inhibitor of all known classes of endopeptidase (serine-, cysteine-, aspartate- and metalloproteases).

The measurement of urinary proteins in the urine allows the diagnosis of proteinuria. In case of a microalbuminuria (selective proteinuria) increased levels (> 150 mg protein/24h) of albumin and transferrin (marker proteins of the tubulus) could be measured. In case of severe diseases of the kidney with pathological changes of the basal membrane in the glomerulus (Chronic Glomerulonephritis, Tubulopathy, Nephrotic Syndrome) the protein level will rise up to > 3 g/24h and also the protein type will change to high molecular proteins like β-Lipoproteins and α-2-Macroglobulin (non-selective proteinuria).

Additionally previous investigations have shown that the determination of the two acute-phase proteins in the urine, C-reactive protein (CRP) and α-2-Macroglobulin, allows a noninvasive diagnosis of acute renal transplant dysfunction and rejection.

Indication

- Marker for glomerulonephropathy
- Monitoring of renal transplant dysfunction and rejection (together with CRP)
3. PRINCIPLE OF THE TEST

In a first incubation step, the α-2-Macroglobulin in the samples is bound to polyclonal rabbit antibodies (in excess), which are immobilized to the surface of the microtitre wells. To remove all unbound substances, a washing step is carried out. In a second incubation step, a Peroxidase-labeled anti α-2-Macroglobulin (POD-Antibody) antibody is added. After another washing step, to remove all unbound substances, the solid phase is incubated with the substrate, tetramethylbenzidine (TMB). An acidic stop solution is then added to stop the reaction. The color converts from blue to yellow. The intensity of the yellow color is directly proportional to the concentration of α-2-Macroglobulin in the sample. A dose response curve of the absorbance unit (optical density, OD) vs. Concentration is generated, using results obtained from the calibrators. α-2-Macroglobulin, present in the patient samples, is determined directly from this curve.

4. MATERIAL SUPPLIED

<table>
<thead>
<tr>
<th>Cat. No</th>
<th>Content</th>
<th>Kit Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>K6610MTP</td>
<td>PLATE</td>
<td>One holder with pre-coated strips</td>
<td>12 x 8 wells</td>
</tr>
<tr>
<td>K6610WB</td>
<td>WASHBUF</td>
<td>ELISA wash buffer concentrate (10x)</td>
<td>1 x 100 ml</td>
</tr>
<tr>
<td>K6610K</td>
<td>CONJ</td>
<td>Conjugate, (rabbit anti α-2-Macroglobulin peroxidase-labeled)</td>
<td>1 x 300 μl</td>
</tr>
<tr>
<td>K6610ST</td>
<td>STD</td>
<td>Calibrators, lyophilized</td>
<td>6 vials</td>
</tr>
<tr>
<td>K6610KO1</td>
<td>CTRL</td>
<td>Control, lyophilized</td>
<td>1 vial</td>
</tr>
<tr>
<td>K6610KO2</td>
<td>CTRL</td>
<td>Control, lyophilized</td>
<td>1 vial</td>
</tr>
<tr>
<td>K6610VP</td>
<td>SAMPLEBUF</td>
<td>Sample dilution buffer, ready-to-use</td>
<td>1 x 100 ml</td>
</tr>
<tr>
<td>K6610NaCl</td>
<td>NACL</td>
<td>0.9 % NaCl-solution, ready-to-use</td>
<td>25 ml</td>
</tr>
<tr>
<td>K6610TMB</td>
<td>SUB</td>
<td>TMB substrate (Tetramethylbenzidine), ready-to-use</td>
<td>2 x 15 ml</td>
</tr>
<tr>
<td>K6610AC</td>
<td>STOP</td>
<td>ELISA stop solution, ready-to-use</td>
<td>1 x 15 ml</td>
</tr>
</tbody>
</table>
5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- Precision pipettors calibrated to deliver 10-200 μl
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 3000 x g
- Vortex-Mixer
- Microplate reader 450 nm

*Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 μm) with an electrical conductivity < 0.055 μS/cm at 25°C (≥18.2 MΩ cm).

6. PREPARATION AND STORAGE OF REAGENTS

- To run the assay more than one time, please make sure that the reagents are carefully stored as mentioned. Prepare just the appropriate amount necessary for the assay.
- The ELISA wash buffer concentrate (WASHBUF) should be diluted with ultra pure water 1:10 before use (add 450 ml ultra pure water to 50 ml concentrate). Crystals could occur due to high salt concentration. The crystals have to be resuspended before dilution of the buffer solutions using a water bath (37°C). The buffer concentrates are stable at 2-8°C until the expiry date stated on the label. Diluted solutions could be stored at 2-8°C for 1 month.
- The reagent tubes with the lower amounts should be centrifuged before use, to guarantee that the required amount is available for use.
- The calibrators (STD) and controls (CTRL) must be reconstituted with 250μl of ultra pure water. Allow the vial to stand for 10 minutes and then mix thoroughly by gentle inversion to ensure complete reconstitution. Reconstituted calibrators and controls are stable at -20°C until the expiry date stated on the label.
- The Conjugate (CONJ; POD antibody) has to be diluted 1:101 in ELISA wash buffer (100 μl POD antibody and 10 ml ELISA wash buffer). The undiluted antibody is stable at 2-8°C until expiry date given on the label. Diluted antibody solution is not stable and could not be stored.
7. PRECAUTIONS

- For *in vitro* diagnostic use only.
- The calibrators and controls contain human source material which was tested and found to be non-reactive to HBsAg, anti-HIV-1/2, and anti-HCV. Since no method can offer complete assurance that hepatitis B virus, HIV-1/2, HVC or other infectious agents are absent, these reagents should be handled as if potentially infectious.
- Stop Solution consists of diluted Sulfuric Acid. This is a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation.
- Reagents should not be used beyond the expiration date shown on kit label.

8. SPECIMEN COLLECTION AND PREPARATION

**Plasma and serum**

Plasma and sera must be diluted 1:2000 with SAMPLEBUF (sample dilution buffer).

For example:

- 30 μl sample + 270 μl SAMPLEBUF, mix well (dilution I) (1:10)
- 50 μl of dilution I + 450 μl SAMPLEBUF, mix well (dilution II) (1:10)
- 50 μl of dilution II + 950 μl SAMPLEBUF, mix well (dilution III) (1:20)

For analysis, pipette 10 μl of dilution step III solution per well.

Plasma and sera are stable at 2-8°C for about 14 days. For long time storage we recommend –20°C.

**Urine**

Urine samples can be measured directly.

9. ASSAY PROCEDURE

*Procedural notes*

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore, we recommend not to assemble wells of different microtiter plates for analysis, even if they are of the same batch.
- The quality control guidelines should be observed.
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- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the test results. Immundiagnostik can therefore not be held reliable for any damage resulting from this.
- Carry out the assay with the actual manual delivered with the kit.

**Test procedure**

Wash the precoated microtiter plate 5 x with 250 μl ELISA wash buffer (WASHBUF). Carry out the tests in duplicate.

1. Add 200 μl 0.9% NACL (NaCl) solution into each well.
2. Add 10 μl of each STD (standard), CTRL (controls) and SAMPLE (patient samples).
3. Cover the plate tightly and incubate for 1 hour at room temperature (18-26°C) shaking on a horizontal mixer or for 2 hours at room temperature (18-26°C) without any shaking.
4. Decant the content of the plate and wash the wells 5 x with 250μl ELISA WASHBUF (wash buffer).
5. Add 200 μl prediluted CONJ (Conjugate).
6. Incubate for 1 hour, shaking on a horizontal mixer, at room temperature.
7. Decant the content of the plate and wash the wells 5 x with 250μl ELISA WASHBUF.
8. Add 200 μl SUB (TMB substrate) solution.
9. Incubate for 10-20 minutes at room temperature.
10. Add 50 μl STOP (stop solution) and mix shortly.
11. Determine absorption with an ELISA reader at 450 nm against 620 nm as reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the measurement range of the photometer, absorption must be measured immediately at 405 nm against 620 nm as reference.
10. RESULTS

A calibration curve is constructed from the standards. Commercially available software can be used as well as graph paper. Results of the samples are read from this calibration curve. THE CALIBRATION CURVE IS NOT LINEAR, therefore a spline- or 4PL algorithm is recommended.

**Typical calibration curve**

<table>
<thead>
<tr>
<th>Concentration [mg/l]</th>
<th>5</th>
<th>1.66</th>
<th>0.56</th>
<th>0.19</th>
<th>0.07</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD mean value</td>
<td>1.711</td>
<td>1.021</td>
<td>0.501</td>
<td>0.260</td>
<td>0.160</td>
<td>0.148</td>
</tr>
</tbody>
</table>

The data is for demonstration only and cannot be used in place of data generations at the time of the assay.
Plasma and Serum

The measured plasma and serum concentration have to be multiplied with 2000 to get the right concentration.

11. LIMITATIONS

Samples with α-2-Macroglobulin levels greater than the highest calibrator, should be diluted and re-assayed.

12. QUALITY CONTROL

Immundiagnostik recommends commercial control samples for internal quality control.

Control samples should be analyzed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid, if within the same assay one or more values of the quality control sample are outside the acceptable limits.

Expected values

Plasma and serum: 1.3 – 3.0 g/l
Urine: < 0.18 mg/l

We recommend each laboratory to establish its own norm concentration range.
13. PERFORMANCE CHARACTERISTICS

**Precision and reproducibility**

The intra-assay variation of the Immundiagnostik α₂-Makroglobulin ELISA test was calculated from 32 determinations on each one of two samples.

**Intra-Assay CV n= 32**

<table>
<thead>
<tr>
<th>Sample</th>
<th>α₂-Makroglobulin mean value [mg/L]</th>
<th>Intra-Assay CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1125.53</td>
<td>6.20</td>
</tr>
<tr>
<td>2</td>
<td>676.92</td>
<td>7.96</td>
</tr>
</tbody>
</table>

The inter-assay variation of the Immundiagnostik α₂-Makroglobulin ELISA test was calculated from data on 2 samples obtained in 10 different assays by three technicians on two different lots of reagents over a period of three months.

**Inter-Assay CV n= 10**

<table>
<thead>
<tr>
<th>Sample</th>
<th>α₂-Makroglobulin mean value [mg/L]</th>
<th>Inter-Assay CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1937.12</td>
<td>9.23</td>
</tr>
<tr>
<td>2</td>
<td>1298.07</td>
<td>8.85</td>
</tr>
</tbody>
</table>
Recovery
Two samples were spiked with three different \( \alpha_2 \)-Makroglobulin-concentrations and measured with the assay.
Recovery n=3

<table>
<thead>
<tr>
<th>Sample [ng/ml]</th>
<th>Spike [ng/ml]</th>
<th>( \alpha_2 )-Makroglobulin expected [ng/ml]</th>
<th>( \alpha_2 )-Makroglobulin measured [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>344</td>
<td>521</td>
<td>865</td>
<td>736</td>
</tr>
<tr>
<td>344</td>
<td>415</td>
<td>759</td>
<td>730</td>
</tr>
<tr>
<td>344</td>
<td>293</td>
<td>567</td>
<td>585</td>
</tr>
<tr>
<td>176</td>
<td>521</td>
<td>697</td>
<td>571</td>
</tr>
<tr>
<td>176</td>
<td>415</td>
<td>591</td>
<td>540</td>
</tr>
<tr>
<td>176</td>
<td>293</td>
<td>469</td>
<td>420</td>
</tr>
</tbody>
</table>

Sensitivity
The sensitivity was set as \( B_0 + 2SD \). The zero-standard was measured 22 times.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \alpha_2 )-Makroglobulin mean value [OD]</th>
<th>Standard variation</th>
<th>Detection limit [mg/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.019</td>
<td>0.011</td>
<td>0.058</td>
</tr>
</tbody>
</table>
**Sample dilution**

Linearity n= 2

Two patient serum samples were diluted with sample dilution buffer. The results are shown below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Expected [mg/L]</th>
<th>Measured [mg/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1:2000</td>
<td>2528.3</td>
<td>2528.3</td>
</tr>
<tr>
<td></td>
<td>1:4000</td>
<td>1264.2</td>
<td>1346.1</td>
</tr>
<tr>
<td></td>
<td>1:8000</td>
<td>632.1</td>
<td>675.9</td>
</tr>
<tr>
<td></td>
<td>1:16000</td>
<td>316.0</td>
<td>343.4</td>
</tr>
<tr>
<td>B</td>
<td>1:2000</td>
<td>2036.8</td>
<td>2036.8</td>
</tr>
<tr>
<td></td>
<td>1:4000</td>
<td>1018.4</td>
<td>1046.6</td>
</tr>
<tr>
<td></td>
<td>1:8000</td>
<td>509.2</td>
<td>509.2</td>
</tr>
<tr>
<td></td>
<td>1:16000</td>
<td>254.6</td>
<td>285.4</td>
</tr>
</tbody>
</table>

**14. REFERENCES**

15. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.

- The test components which are made of human serum are tested for Australia antigen and HIV and found to be negative. However, since no test method can offer complete assurance that infectious agents are absent; these reagents should be handled as recommended for any potentially infectious human serum or blood specimen. The normal precautions for laboratory working should be observed.

- Reagents of the test package contain sodium azide as a bactericide. Contact with skin or mucous membranes has to be avoided.

- All reagents in the test package are to be used for in-vitro diagnostics only.

- The reagents should not be used after the date of expiry (see label on the test package).

- The guidelines for medical laboratories should be observed.

- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the results of the test. Immundiagnostik can therefore not be held reliable for any damage resulting from.
Manual

α2-Macroglobulin

Used symbols:

- Temperature limitation
- Catalogue Number
- In Vitro Diagnostic Medical Device
- Contains sufficient for <n> tests
- Manufacturer
- Use by
- Lot number

For reference use only.
For research use only.