Instructions for use

Calretinin ELISA

Enzym Immunoassay for the Quantitative Determination of Calretinin in Plasma and Serum

RUO
For Research Use Only
Not for Use in Diagnostic Procedures

REF EA611/96
12 x 9
2 – 8 °C
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Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>Content</td>
</tr>
<tr>
<td>LOT</td>
<td>Batch code</td>
</tr>
<tr>
<td>Manufacturer</td>
<td></td>
</tr>
<tr>
<td>REF</td>
<td>Catalogue number</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expiry Date</td>
</tr>
<tr>
<td></td>
<td>Temperature limitation</td>
</tr>
<tr>
<td></td>
<td>Sufficient for ... determinations</td>
</tr>
<tr>
<td></td>
<td>Consult instructions for use</td>
</tr>
</tbody>
</table>
1. Introduction and Principle of the Test

For some time now, the production and application of asbestos has been banned in more than 55 countries in the world. However, the number of asbestos-associated cancers – mainly malignant lung cancer and mesothelioma – is still high.

Due to long latency of the disease and ongoing production and application of asbestos in several countries, no substantial improvement is expected. Detecting tumors early – preferably at stages without clinical symptoms - might improve the chances for a curative therapy.

In collaboration with scientists of the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance – Institute of the Ruhr-University Bochum (IPA) the DLD Diagnostika GmbH developed a promising method based on calretinin as a biomarker for early detection – especially of mesothelioma – in plasma and serum samples.

Currently, calretinin is one of the best available markers for the detection of mesothelioma.

The calretinin ELISA is a sandwich enzyme immunoassay utilizing a purified rabbit polyclonal antibody. During incubation, calretinin from diluted patient samples binds to a calretinin antibody (capture antibody) immobilized on the surface of microtiter plate wells. After a washing step, a biotinylated calretinin antibody (detection antibody) is added that binds to the captured calretinin of the patient sample.

After a second washing step, conjugated streptavidin peroxidase is added, binding specifically to the biotinylated detection antibody. Following a third washing step, the bound amount of enzyme – equivalent to the amount of calretinin – is quantified via the turnover of the substrate tetramethylbenzidin (TMB).

During the enzyme reaction, a blue dye is generated. Addition of sulfuric acid stops the reaction and causes the solution to turn yellow.

The extinction of the samples is measured using a microtiter plate reader at 450 nm (reference wavelength between 570 nm and 650 nm) and the concentration of calretinin is calculated using the standards and controls of the assay.
2. **Precautions**
   - For in vitro use only.
   - Disposable gloves and safety glasses should be used.
   - All reagents of human origin used in this kit are tested for HIV I/II antibodies, HCV and HBsAg and found to be negative. However, because no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as potentially biohazardous materials.
   - Material of animal origin used in the preparation of the kit have been obtained from certified healthy animals but these materials should be handled as potentially infectious.
   - Some components of this kit are containing hazardous reagents. These components are marked with the adequate hazard label.

3. **Storage and Stability**
   On arrival, store the kit at 2-8 °C. Once opened the kit is stable until its expiry date. For stability of prepared reagents refer to Preparation of Reagents. Do not use components beyond the expiration date shown on the kit labels. Do not mix various lots of any kit component within an individual assay.

4. **Contents of the Kit**

4.1 **MT-Strips**
   - 8 wells each, break apart
   - precoated with calretinin antiserum

4.2 **Standards 1 - 6**
   - lyophilized, dissolve in 200 µl aqua dist.;
   - concentrations; see qc certificate
   - Concentrations:
   
<table>
<thead>
<tr>
<th>Standard</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/ml</td>
<td>0</td>
<td>0.25</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>
4.3 **Control 1 & 2** \(\text{CON 1 & 2}\) 2 vials
Lyophilized, dissolve in 200 µl aqua dist.; concentrations; see qc certificate
Range: see q.c. certificate

4.4 **Diluent** \(\text{Diluent}\) 1 vial
7 ml, color coded yellow, ready for use

4.5 **Antiserum** \(\text{AS}\) 1 vial
6 ml, ready for use, color coded blue rabbit-anti-Calretinin

4.6 **Enzyme Conjugate** \(\text{CONJ}\) 1 vial
0.15 ml, 200x concentrated Streptavidin-peroxidase

4.7 **Enzyme Conjugate Buffer** \(\text{CONJ-BUFF}\) 1 vial
18 ml, ready for use

4.8 **Wash Buffer** \(\text{WASH}\) 1 vial
20 ml, 50x concentrated
Dilute content with distilled water to 1 litre total volume

4.9 **Substrate** \(\text{SUB}\) 1 vial
13 ml TMB solution, ready for use

4.10 **Stop Solution** \(\text{STOP}\) 1 vial
13 ml, ready for use
contains 0.3 M sulphuric acid

4.11 **Preparation Plate** \(\text{PLATE}\) 1 plates
for dilution

4.12 **Adhesive Foil** \(\text{FOIL}\) 6 pieces
Ready for use

4.13 **Dilution Vial** \(\text{DILUTION-VIAL}\) 3 pieces
for dilution of Enzyme Conjugate (max. 14 ml)
Additional materials and equipment required but not provided:

- Pipettes 15, 50, 60 and 100 µl
- Orbital shaker
- Multichannel pipette or Microplate washing device
- Eppendorf Multipette (or similar devices)
- Microplate photometer (450 nm)
- Centrifuge (2,500 g)
- Distilled water

5. Specimen Collection and Storage

The test can be performed with EDTA plasma and serum.

**EDTA plasma and serum**

EDTA plasma and serum should be used. Haemolytic and lipaemic samples should not be used.

The samples can be stored up to 12 hours at 2 - 8 °C. For a longer storage (up to 24 months) the samples must be frozen at -20 °C. Repeated freezing and thawing of samples should be avoided.
6. Preparation of Reagents

Standards and Controls

Dissolve standards and controls with 200 µl dist. water each, leave for minimum 30 minutes on a roller mixer or orbital shaker and vortex until contents is completely dissolved (visual check). Handle with care in order to minimize foam formation.
The reconstituted standards and controls should be stored frozen at -20 °C and are stable until expiry date printed on vial label.

Enzyme Conjugate

Do not vortex!
Centrifuge kit vial for 5 minutes at 2,000 g. Pipette needed volume from supernatant into a plastic vial (PP, PE) and dilute 200-fold with Enzyme Conjugate Buffer in a Dilution Vial (see 4.13, max. 14 ml).
For example: dilute 30 µl Enzyme Conjugate with 6 ml Enzyme Conjugate Buffer. This is sufficient for 6 strips.
Leaves for minimum 30 minutes on a roller mixer or orbital shaker, avoid excessive foam formation. Do not vortex!
Discard remains after use.

Wash Buffer

Dilute the content with dist. water to a total volume of 1 litre and mix shortly. The diluted wash buffer can to be stored at 2 - 8 °C for a maximum period of 4 weeks.
However, when not using up the kit in one run, it is recommended to prepare only the required amount of wash buffer.

All other reagents are ready for use.
7. **Test Procedure**

Allow reagents and samples to reach room temperature. Determinations in duplicates are recommended.

7.1. **Dilution of samples**

1. Pipette 15 µl standard 1 - 6, control 1 & 2 and plasma / serum samples into the respective wells of the preparation plate.
2. Pipette each 60 µl Diluent into all wells.
3. Incubate for 60 minutes at room temperature on an orbital shaker with medium frequency. Cover the wells or the plate. Take each 50 µl for the ELISA.
7.2  **ELISA Procedure**

Allow reagents and samples to reach room temperature.

1. Pipette each 50 µl diluted Standards 1 to 6, Controls and Samples into the respective wells of the coated microtiter strips.

2. Cover the plate with adhesive foil and incubate for 2 hours at room temperature (20 – 25 °C) on an orbital shaker with medium frequency.

3. Discard or aspirate the contents of the wells, add each 300 µl Wash Buffer, again discard or aspirate the contents of the wells. Remove residual liquid by tapping the inverted plate on clean absorbent paper. Repeat the washing procedure 4 times.

4. Pipette each 50 µl antiserum into all wells.

5. Cover plate with foil and incubate for 60 minutes at room temperature on an orbital shaker with medium frequency.


7. Pipette each 100 µl diluted enzyme conjugate into all wells.

8. Cover plate with foil and incubate for 60 minutes at room temperature on an orbital shaker with medium frequency.


10. Pipette each 100 µl Substrate into all wells.

11. Incubate for 25 +/- 5 minutes at room temperature (20 - 25 °C) on an orbital shaker with medium frequency.

12. Pipette each 100 µl Stop Solution into all wells. Shake on a horizontal shaker for 10 seconds.

13. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.
8. **Calculation of the Results**

On a semilogarithmic graph paper the concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). Alternatively, the optical density of each standard and sample can be related to the optical density of the zero standard, expressed as the ratio OD/OD_{max}, and then plotted on the y-axis.

The concentration of the controls and plasma/serum samples can be read directly from this standard curve in ng/ml.

**Typical standard curve:**

![Graph showing the typical standard curve for Calretinin ELISA](Image)

\[
y = \frac{(A - D)}{1 + \left(\frac{x}{C}\right)^B} + D
\]

<table>
<thead>
<tr>
<th>Std (Standards: Concentration vs MeanValue)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,198</td>
<td>1,169</td>
<td>4,029</td>
<td>4,74</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
9. **Assay Characteristics**

9.1 **Reference Range**

<table>
<thead>
<tr>
<th>EDTA-Plasma, Serum</th>
<th>Reference Range Men</th>
<th>Reference Range Woman</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 0.6 ng/ml</td>
<td>&lt; 0.8 ng/ml</td>
</tr>
</tbody>
</table>

The reference range given above should only be taken as a guideline. It is recommended that each laboratory should establish its own normal values.

9.2 **Sensitivity**

The lower limit of detection was determined by taking the 2-fold standard deviation of the absorbance of the Zero Reference and reading the corresponding value from the standard curve.

<table>
<thead>
<tr>
<th>EDTA-Plasma; Serum</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 ng/ml</td>
</tr>
</tbody>
</table>

9.3 **Linearity**

<table>
<thead>
<tr>
<th>Range (ng/ml)</th>
<th>Highest Dilution</th>
<th>Mean (%)</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.47 - 3.03</td>
<td>1 : 7 with dist. water</td>
<td>102</td>
<td>108 - 95</td>
</tr>
</tbody>
</table>

9.4. **Reproducibility**

The reproducibility of the ELISA method was investigated by measuring the intra- and inter-assay-coefficients of variation (cv).

Concentrations in ng/ml

Plasma, serum

<table>
<thead>
<tr>
<th>Range (ng/ml)</th>
<th>Intra-Assay-cv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.64 – 2.00</td>
<td>8.1 – 6.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Range (ng/ml)</th>
<th>Inter-Assay-cv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.57 – 1.54</td>
<td>10.4 – 10.0</td>
</tr>
</tbody>
</table>
10. Literature


10. Changes to declare

Cal-d_13: The hazardous substance symbols have been removed from the Enzyme Conjugate and Enzyme Conjugate Buffer; however, the composition has not been changed. Section 6 Wash Buffer: The preparation is described in more detail and storage of the prepared Wash Buffer at -20°C has been removed. Section 6. Enzyme Conjugate: the centrifugal force specification has been reduced from 4000 x g to 2000 x g. Section 7.2 point 10 and the pipetting scheme have been supplemented with a 10 second shaking step.
Pipetting Scheme Sample Preparation

<table>
<thead>
<tr>
<th></th>
<th>Standards</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 1 - 6</td>
<td>µl</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Control 1 &amp; 2</td>
<td>µl</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Sample</td>
<td>µl</td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

Diluent µl 60 60 60

cover the plate with foil
shake 60 minutes at room temperature

take each 50 µl for the ELISA
Pipetting Scheme ELISA

<table>
<thead>
<tr>
<th></th>
<th>Standards</th>
<th>Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dil. Standard 1 - 6 µl</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dil. Control 1 &amp; 2 µl</td>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Diluted Sample µl</td>
<td></td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

cover the plate with adhesive foil  
shake for 2 hours at room temperature  

4 x washing

<table>
<thead>
<tr>
<th></th>
<th>50</th>
<th>50</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiserum µl</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

cover the plate with adhesive foil  
shake for 60 minutes at room temperature  

4 x washing

<table>
<thead>
<tr>
<th></th>
<th>100</th>
<th>100</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Conjugate µl</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

cover the plate with adhesive foil  
shake for 60 minutes at room temperature  

4 x washing

<table>
<thead>
<tr>
<th></th>
<th>100</th>
<th>100</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate µl</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

shake for 25 ± 5 minutes at room temperature

Stop Solution µl  
shaking for 10 seconds  
reading of absorbance at 450 nm