DIAGNOSTIKA GMBH

Instruction for use

Calretinin ELISA

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

For research use only

REF EA611/96

12 x 8

2 – 8 °C

DLD Gesellschaft für Diagnostika und medizinische Geräte mbH
Adlerhorst 15 • 22459 Hamburg • Germany
Tel +49-40-555 87 10 • Fax +49-40-555 87 111
Internet: http://www.dld-diagnostika.de • E-Mail: contact@dld-diagnostika.de

Contents

Symbols

CONT	Content		Use by
LOT	Batch code		Temperature limitation
	Manufacturer		Sufficient for ... determinations
REF	Catalogue number		Consult instructions for use

Hazard Pictograms

Danger

Warning

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** **STRIPS** 12 strips
8 wells each, break apart
precoated with calretinin antiserum

4.2 **Standards 1 - 6** **CAL 1-6** 6 vials
lyophilized, dissolve in 200 µl aqua dist.;
concentrations; see qc certificate

Concentrations:

Standard	1	2	3	4	5	6
ng/ml	0	0.25	0.5	1.0	2.0	4.0

4.3 **Control 1 & 2** **CON 1 & 2** 2 vials
lyophilized, dissolve in 200 µl aqua dist.;
concentrations; see qc certificate
Range: see q.c. certificate

4.4 **Diluent** **Diluent** 1 vial
7 ml, color coded yellow, ready for use

4.5 **Antiserum** **AS** 1 vial
6 ml, ready for use, color coded blue
rabbit-anti-Calretinin

4.6 **Enzyme Conjugate** **CONJ**  Warning 1 vial
0.15 ml, 200x concentrated
goat anti-rabbit-IgG-peroxidase

4.7 **Enzyme Conjugate Buffer** **CONJ-BUFF**  Warning 1 vial
18 ml, ready for use

4.8 **Wash Buffer** **WASH** 1 vial
20 ml, 50x concentrated
Dilute content with distilled water
to 1 litre total volume

4.9 **Substrate** **SUB**  Danger 1 vial
13 ml TMB solution, ready for use

4.10 **Stop Solution** **STOP** 1 vial
13 ml, ready for use
contains 0.3 M sulphuric acid

4.11 **Preparation Plate** **PLATE** 1 plates
for dilution

4.12 **Adhesive Foil** **FOIL** 6 pieces
Ready for use

4.13 **Dilution Vial** **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 **CAL 1 – 6** **CON 1 & 2**

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 **CONJ**

Do not vortex!
Centrifuge kit vial for 5 minutes at 4,000 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.
Leave for minimum 30 minutes on a roller mixer or orbital shaker, avoid excessive foam formation. Do not vortex!
Discard remains after use.

Wash Buffer **WASH**

Dilute the content with dist. water to a total volume of 1 litre.
The diluted wash buffer has to be stored at 2 - 8 °C for a maximum period of 4 weeks. For longer storage freeze at -20 °C.

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.

6. Washing: Repeat step 3.

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.

9. Washing: Repeat step 3.

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.

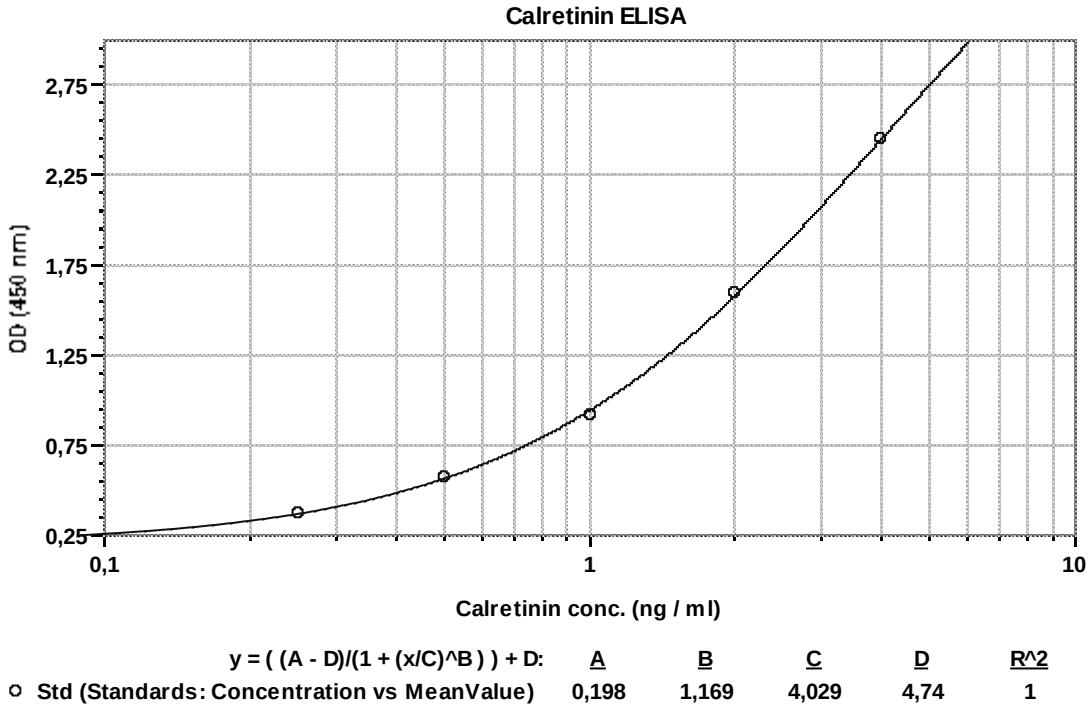
13. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.

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:



9. Assay Characteristics

9.1 Reference Range

	Reference Range
EDTA-Plasma, Serum	< 0.8 ng/ml

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 2fold standard deviation of the absorbance of the Zero Reference and reading the corresponding value from the standard curve.

	Sensitivity
EDTA-Plasma; Serum	0.05 ng/ml
Calculation	OD Cal1 + 2 sd

9.3 Linearity

Range (ng/ml)	Highest Dilution	Mean (%)	Range (%)
0.47 - 3.03	1 : 7 with dist. water	102	108 - 95

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

Range (ng/ml)	Intra-Assay-cv (%)
0.64 – 2.00	8.1 – 6.6

Range (ng/ml)	Inter-Assay-cv (%)
0.57 – 1.54	10.4 – 10.0

10. Literature

Johnen G, Burek K, Raiko I, Wichert K, Pesch B, Weber DG, Lehnert M, Casjens S, Hagemeyer O, Taeger D, Brüning T, MoMar Study Group. Prediagnostic detection of mesothelioma by circulating calretinin and mesothelin – a case-control comparison nested into a prospective cohort of asbestos-exposed workers. Scientific Reports 2018; 8: 14321

Johnen G., Gawrych K., Raiko I., Casjens S., Pesch B., Weber D. G., Taeger D., Lehnert M., Kollmeier J., Bauer T., Musk A. W., Robinson B. W. S., Brüning T., Creaney J.
Calretinin as a blood-based biomarker for mesothelioma
BMC Cancer 2017; 17: 386

Casjens S, Weber DG, Johnen G, et al.
Assessment of potential predictors of calretinin and mesothelin to improve the diagnostic performance to detect malignant mesothelioma: results from a population based cohort study.
BMJ Open 2017;7:e017104.doi:10.1136/bmjopen-2017-017104

Raiko I, Sander I, Weber DG, Raulf-Heimsoth M, Gillissen A, Kollmeier J, Scherpereel A, Brüning T, Johnen G.
Development of an enzyme-linked immunosorbent assay for the detection of human calretinin in plasma and serum of mesothelioma patients.
BMC Cancer 2010; 10: 242

Pesch B, Brüning T, Johnen G, Casjens S, Bonberg N, Taeger D, Müller A, Weber DG, Behrens T.
Biomarker research with prospective study designs for the early detection of cancer.
Biochim Biophys Acta 2014; 1844: 874-883

Calretinin ELISA: A New Assay for the Detection of Mesothelioma in Blood Samples.
Georg Johnen**,** Irina Raiko, Ingrid Sander, Daniel G. Weber, Monika Raulf-Heimsoth, Adrian Gillissen, Jens Kollmeier, Klaus-Michael Müller, Arnaud Scherpereel, Thomas Brüning (data on file)

Pipetting Scheme Sample Preparation

		Standards	Control	Sample
Standard 1 - 6	µl	15		
Control 1 & 2	µl		15	
Sample	µl			15

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

		Standards	Control	Sample
Dil. Standard 1 - 6	µl	50		
Dil. Control 1 & 2	µl		50	
Diluted Sample	µl			50

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

4 x washing

Antiserum	µl	50	50	50

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

4 x washing

Enzyme Conjugate	µl	100	100	100

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

4 x washing

Substrate	µl	100	100	100

shake for 25 ± 5 minutes at room temperature

Stop Solution	µl	100	100	100

reading of absorbance at 450 nm