DIAGNOSTIKA GMBH

Instructions for Use

Calretinin ELISA

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

CE IVD

Item No	EA611/96
Σ	12 x 8
	2 – 8 °C

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Table of Contents

Symbols

IVD	In Vitro Diagnostic Medical Device		
CONT	Content	CE	EC Declaration of conformity
LOT	Lot Number		Store at
	Manufactured by		Sufficient for ... determinations
REF	Catalogue Number of Manufacturer		Consult Instructions for Use

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 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 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 diagnostic use only. For professional use only.
- 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.
- 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.
- Individual components of different lots and test kits should not be interchanged. The expiry dates and storage conditions stated on the packaging and the labels of the individual components must be observed.
- When handling the reagents, controls and samples, the current laboratory safety guidelines and good laboratory practice should be observed.
- Wear protective clothing, disposable gloves, and safety goggles while performing the test.
- Avoid any actions that could result in ingestion, inhalation or injection of the reagents. Never pipette by mouth.
- Avoid contact with individual reagents, these can cause irritation and chemical burns.
- Dispose of waste according to state and local environmental protection regulations.
- The quality control guidelines in the medical laboratory regarding the inclusion of control samples and/or pooled samples should be observed.

3 Storage and Stability

Upon arrival, store the kit at 2 – 8 °C to keep it stable until its expiry date. Once opened the kit is stable until its expiry date. The shelf life of the ready-to-use reagents is indicated on the respective bottle label. For stability of prepared reagents refer to 6.

Reagents must equilibrate to room temperature before use and refrigerated immediately after use.

4 Contents of the Kit

MT-Strips **STRIPS** 12 strips

8 wells each, break apart, precoated with calretinin antiserum

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	2	4

Controls 1 & 2 **CON 1 & 2** 2 vials

Lyophilized

dissolve in 200 µl aqua dist., for concentrations & range see QC certificate

Diluent **DILUENT** 1 vial

7 ml, color coded yellow, ready for use

Antiserum **AS** 1 vial

6 ml, color coded blue, ready for use, rabbit-anti-calretinin

Enzyme Conjugate **CONJ** 1 vial

0.15 ml, 200x concentrated, Streptavidin-peroxidase

Enzyme Conjugate Buffer **CONJ-BUFF** 1 vial

18 ml, ready for use

Wash Buffer **WASH** 1 vial

20 ml, 50x concentrated,

Dilute content with dist. Water to 1 litre total volume

Substrate **SUB** 1 vial

13 ml TMB Lösung, ready for use

Stop Solution **STOP** 1 vial

13 ml, ready for use, contains 0.3M sulphuric acid

Preparation Plate **PLATE** 1 plate

For dilution of samples

Adhesive Foil **FOIL** 6 pieces

Ready for use

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

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

The samples can be stored up to 24 hours at room temperature. 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

6.1 Standards and Controls

Dissolve standards CAL 1 – 6 and controls CON 1 & 2 with 200 µl dist. water each, leave for minimum 30 minutes on a roller mixer or orbital shaker and vortex until contents are 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.

6.2 Enzyme Conjugate

Do not vortex!

Centrifuge Enzyme Conjugate CONJ vial for 5 minutes at 2,000 g. Pipette needed volume from supernatant into a Dilution Vial DILUTION-VIAL (max. 14 ml) and dilute 200-fold with Enzyme Conjugate Buffer CONJ-BUFF.

For example: dilute 30 µl Enzyme Conjugate CONJ with 6 ml Enzyme Conjugate Buffer CONJ-BUFF. 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.

6.3 Wash Buffer

Dilute the content of WASH 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

When performing 2 to 3 runs with the kit, it is recommended to prepare only the required amount of wash buffer for each run.

All other reagents are ready for use.

7 Test Procedure

Allow reagents and samples to reach room temperature. Determinations in duplicates are recommended. It is recommended to mark (with permanent marker) the wells of the preparation plate PLATE used for the dilutions to prevent from using them again.

7.1 Dilution of samples

1. Pipette **15 µl of standard 1 – 6** CAL 1 – 6, **control 1 & 2** CON 1 & 2 and of the **samples** into the respective wells of the preparation plate PLATE.
2. Pipette **60 µl Diluent** DILUENT into each well.
3. Cover the wells or the plate with FOIL. Incubate for 60 minutes at room temperature on an orbital shaker with medium frequency.

   Take each 50 µl for the ELISA.

7.2 ELISA Procedure

1. Transfer **50 µl each of diluted Standards, Controls and Samples** into the respective wells of the coated microtiter strips [STRIPS].
2. Cover the plate with adhesive foil [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 diluted Wash Buffer** [WASH], 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.
   Alternatively, a washing device may be used.
4. Pipette **50 µl Antiserum** [AS] into each well.
5. Cover plate with foil [FOIL] and incubate for 60 minutes at room temperature on an orbital shaker with medium frequency.
6. Washing: Repeat step 3.
7. Pipette **100 µl diluted Enzyme Conjugate** (s. 6.2) into each well.
8. Cover plate with foil [FOIL] and incubate for 60 minutes at room temperature on an orbital shaker with medium frequency.
9. Washing: Repeat step 3.
10. Pipette **100 µl Substrate** [SUB] into each well.
11. Incubate for 25 ± 5 minutes at room temperature (20 - 25 °C) on an orbital shaker with medium frequency.
12. Pipette **100 µl Stop Solution** [STOP] into each well. 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.

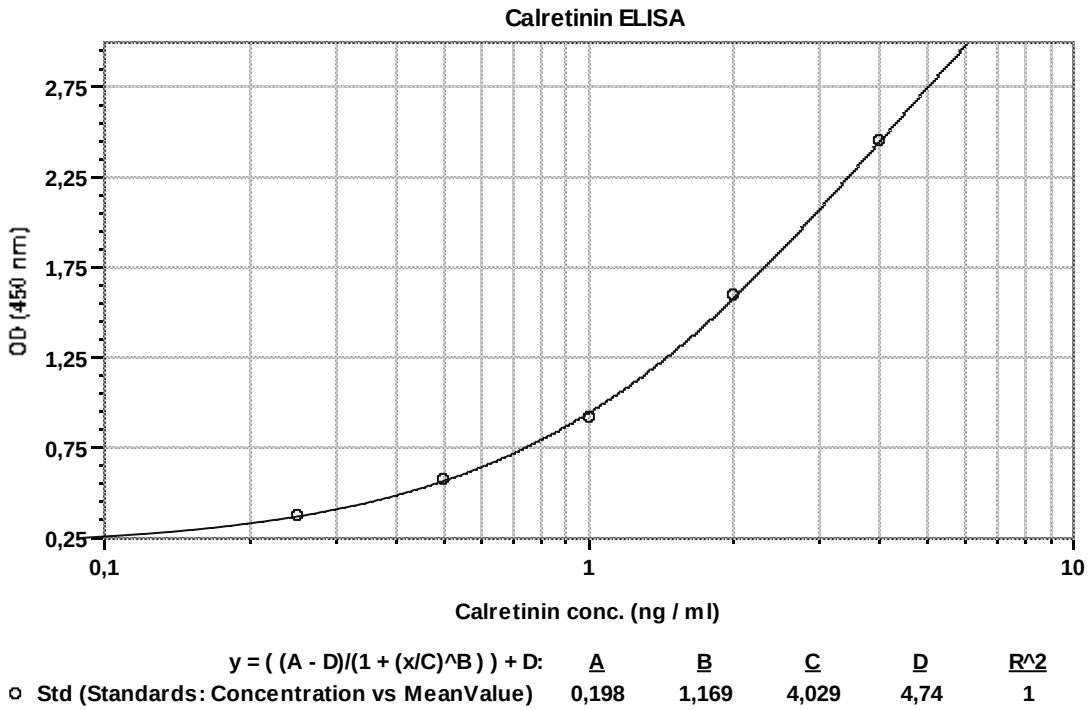
8 Calculation of the Results

The concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear).

When using evaluation software, the 4 Parameter Logistic Regression is recommended (alternatively, Log-Logit or Cubic Spline).

Using their ODs, the concentration of the controls and samples can be read directly from this standard curve in ng/ml.

Typical standard curve:



y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
Std (Standards: Concentration vs MeanValue)	0,198	1,169	4,029	4,74	1

Quality Control: Test results are valid only if the kit controls are within the ranges specified on the QC Certificate. Otherwise, the test should be repeated.

9 Assay Characteristics

9.1 Reference Range

The reference ranges given below should only be taken as a guideline.. It is recommended that each laboratory establishes its own normal values.

	Reference Range Men	Reference Range Woman
EDTA-Plasma, Serum	< 0.6 ng/ml	< 0.8 ng/ml

9.2 Sensitivity

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

9.3 Linearity

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

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

9.5 Calibration

The calibration is carried out by weighing the pure substance.

9.6 Limitations of Method

The result of the Calretinin ELISA is to be seen in connection with other diagnostic procedures and the anamnesis and the resulting questions.

Samples measured above the highest standard must be diluted with distilled water and reassayed. The values of diluted samples must be multiplied by the appropriate dilution factor

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
- Johnen G, Raiko I, Sander I, Weber DG, Raulf-Heimsoth M, Kollmeier J, Gillissen A, Scherpereel A, Müller KM, Brüning T.
  Calretinin – ein vielversprechender Biomarker für die Bestimmung von Mesotheliomen in Blutproben von Asbest-Exponierten.
  IPA-Journal 2011; 02: 22-25
- Marschall V.
  Asbest wird uns noch lange begleiten – Projekt MoMar untersucht über 2.000 Versicherte mit anerkannter BK 4103.
  IPA-Journal 2013; 03: 24-26
- 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

Pipetting Scheme - Sample Preparation

		Standards	Controls	Samples
PLATE:				
CAL 1 - 6	µl	15		
CON 1 & 2	µl		15	
Sample	µl			15
DILUENT	µl	60	60	60

Cover the plate with FOIL and shake 60 minutes at room temperature
Take 50 µl each for the ELISA

Pipetting Scheme - ELISA

		Diluted Standards	Diluted Controls	Diluted Samples
STRIPS:				
Transfer from PLATE into STRIPS:	µl	50	50	50

Cover the plate with FOIL and shake for 2 hours at room temperature
4 x washing (300 µl WASH per well)

AS	µl	50	50	50

Cover the plate with FOIL and
shake for 60 minutes at room temperature
4 x washing (300 µl WASH per well)

Diluted CONJ	µl	100	100	100

Cover the plate with FOIL and
shake for 60 minutes at room temperature
4 x washing (300 µl WASH per well)

SUB	µl	100	100	100

Shake for 25 ± 5 minutes at room temperature

STOP	µl	100	100	100

Shake for 10 seconds
Reading of absorbance at 450 nm (ref. 570 – 670 nm)