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	2 – 8 °C

REF CR00 Wesamin GmbH & Co. KG • Graff 1 • 24568 Oersdorf • Germany
Distributor: DLD Diagnostika GmbH • Adlerhorst 15 • 22459 Hamburg • Germany
Tel. +49 40 5558710 • Fax +49 40 55587111 • contact@dld-diagnostika.de • www.dld-diagnostika.de

Table of Contents

Symbols

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

The symbols of the components of the kit are described in chapter 4 Contents of the test kit.

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 (Calbindin 2, CALB2) 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.
- Before carrying out the test, the valid instructions for use, as included in this kit, should be read completely and the content understood.
- 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.
- Some of the components of this test kit are subject to labeling. These components bear the corresponding hazard symbol on their label. For further information, please see Chapter 4 Contents of the test kit and the relevant safety data sheets.
- Wear protective clothing, disposable gloves, and safety goggles while performing the test.
- 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

The kit is shipped at ambient temperature. 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 and storage 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** - **CAL 6** 6 vials

Lyophilized

dissolve in 200 µl aqua dist. each

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

Controls 1 & 2 **CON 1** & **CON 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  Warning

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

18 ml, ready for use  Warning

Wash Buffer **WASH** 1 vial

20 ml, 50x concentrated,
Dilute content with dist. water to 1 liter total volume, mix briefly

Substrate **SUB** 1 vial

13 ml TMB solution, 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, eventually a thermal shaker
- Multichannel pipette or Microplate washing device
- Eppendorf Multipette (or similar devices)
- Microplate photometer (450 nm)
- Centrifuge (2,000 g)
- Distilled water
- Paper towels, pipette tips, timer

5 Specimen Collection and Storage

Repeated freezing and thawing of samples should be avoided.

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

Centrifuge samples briefly before use.

The samples can be stored up to 12 hours at 2 – 8 °C. For a longer storage (min. 24 months) the samples must be frozen at -20 °C.

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 then 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 the 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 required volume from supernatant into a Dilution Vial DILUTION-VIAL (max. capacity: 14 ml) and dilute 200-fold with Enzyme Conjugate Buffer CONJ-BUFF.

For example: For 6 strips (48 wells) dilute 30 µl Enzyme Conjugate CONJ with 6 ml Enzyme Conjugate Buffer CONJ-BUFF.

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 liter and mix shortly. The diluted wash buffer can 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 21 – 23 °C. Determinations in duplicates are recommended. It is recommended to mark (with a permanent marker) the wells of the preparation plate PLATE used for the dilutions to prevent from using them again.

If a room temperature of 21 - 23 °C cannot be reliably maintained during the test procedure, a thermal shaker should be used.

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 21 - 23 °C room temperature on an orbital shaker with medium frequency (600rpm).

   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 21 - 23 °C on an orbital shaker with medium frequency (600rpm).
3. Discard or aspirate the contents of the wells, add each **300 µl diluted Wash Buffer** [WASH] (see 6.3), 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 21 - 23 °C on an orbital shaker with medium frequency (600rpm).
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 21 - 23 °C on an orbital shaker with medium frequency (600rpm).
9. Washing: Repeat step 3.
10. Pipette **100 µl Substrate** [SUB] into each well.
11. Incubate for 25 ± 5 minutes at 21 - 23 °C on an orbital shaker with medium frequency (600rpm). It is recommended to read the plate at 650nm before stopping the reaction, standard 6 should reach an OD between 9.0 and 1.0.
12. Pipette **100 µl Stop Solution** [STOP] into each well, mix briefly. 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.

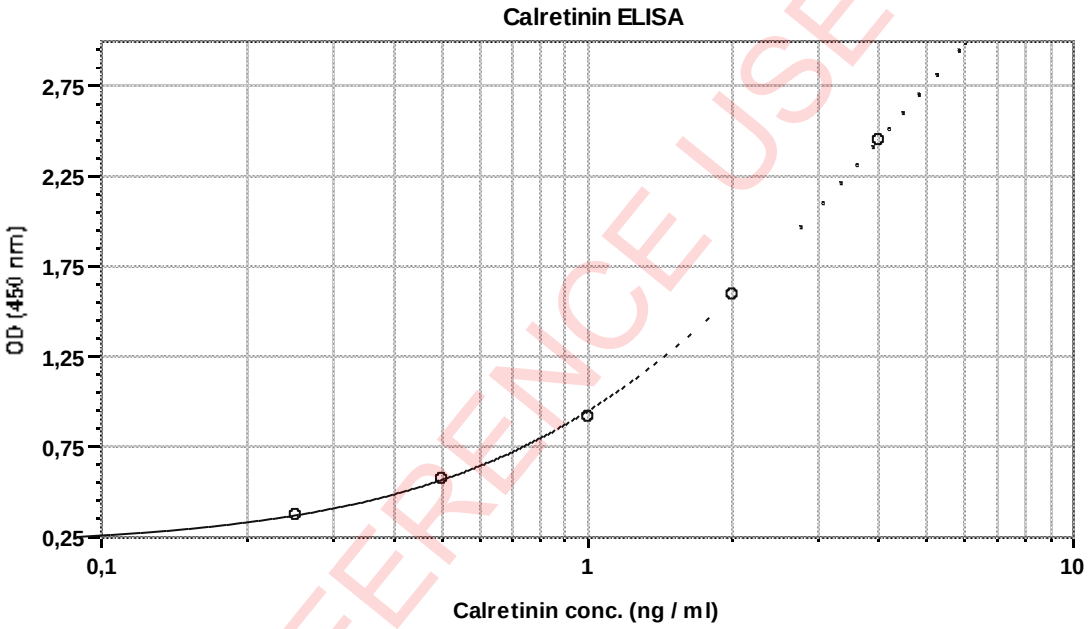
8 Calculation of the Results

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.

Matrix	Reference Range
EDTA-Plasma, Serum	Men < 0.65 ng / ml
EDTA-Plasma, Serum	Women < 1.10 ng / ml

9.2 Sensitivity

Sensitivity	Calculation
0.05 ng/ml	OD $_{Cal1}$ + 2 sd

9.3 Recovery after Spiking

Range (ng / ml)	Mean (%)	Range (%)
0.37 – 2.96	93	90 - 98

9.4 Linearity

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

9.5 Reproducibility

Intra-Assay-CV

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

Inter-Assay-CV

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

9.6 Calibration

The calibration is carried out by weighing the pure substance.

9.7 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 (see 9.4) and re-assayed. 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
- 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
- Weber DG, Casjens S, Wichert K, Lehnert M, Taeger D, Rihs HP, Brüning T, Johnen G.
  MoMar Study Group, Tasks and experiences of the prospective, longitudinal, milticenter MoMar (molecular marcers) study for the early detection of mesothelioma in inviduals formerly exposed to asbestos using liquid biopsies
  Cancers 2023; 15:5896
- Casjens S, Johnen G, Raiko I, Pesch B, Taeger D, Töpfer C, Schonefeld S, Moebus S, Jöckel KH, Brüning T, Weber DG
  Re-evaluation of potential predictors of calretinin and mesothelin in a population-based cohort study using assays for the routine application in clinical medicine
  BMJ Open 2021; 11:e039079
- Hosbach I, Johnen G, Taeger D, Weber D, Brüning T, Wiethege T
  EVA-Mesothel: Früherkennung von Mesotheliomen
  DGUV Forum 2024; 12:24
- Johnen G, Taeger D, Brüning T
  Molekulare Marker zur Krebsfrüherkennung – Follow-up (MoMarFollow)
  IPA-Journal 2022; 3:17

- Johnen G, Burek K, Raiko I, Wichert K, Pesch B, Weber DG, Lehnert M, Casjens S, Hagemeyer O, Taeger D, Brüning T
  MoMar Studiengruppe, Früherkennung von Mesotheliomen mit Biomarkern erstmals möglich – Ergebnisse der MoMar-Studie
  IPA-Journal 2018; 3:16

11 Changes to Declare

Cal-e_17: Changes/additions are highlighted in gray.

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 (21 – 23 °C)

Pipetting Scheme - ELISA

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

Cover the plate with FOIL
Shake for 2 hours at (21 – 23 °C)
4 x washing (300 µl dil. WASH per well)

AS	µl	50	50	50

Cover the plate with FOIL
Shake for 60 minutes at (21 – 23 °C)
4 x washing (300 µl dil. WASH per well)

Diluted CONJ	µl	100	100	100

Cover the plate with FOIL
Shake for 60 minutes at (21 – 23 °C)
4 x washing (300 µl dil. WASH per well)

SUB	µl	100	100	100

Shake for 25 ± 5 minutes at (21 – 23 °C)
(Read at 650 nm to check if OD_{CAL6} at 0.9 - 1.0)

STOP	µl	100	100	100

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