DIAGNOSTIKA GMBH

Instruction for use

Calretinin ELISA

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

R U O **For research use only!**

REF EA611/96

12 x 8

2 – 8 °C

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Contents

1. Introduction and Principle of the Test

2 Million tons of asbestos are still being manufactured annually. Over 100,000 people die each year from cancer diseases caused by exposure to asbestos.

The most common types of asbestos-related cancer diseases are malignant lung tumors and pleural mesothelioma. Asbestos-associated cancers remain on a high level. Blood-based markers are urgently needed to limit invasive diagnostic procedures

Calretinin levels are significantly increased in malignant mesothelioma according to recent studies. The Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA) and DLD have developed a new Sandwich ELISA for the early detection of human calretinin.

The microtiter plate of the Calretinin ELISA is coated with a capture antibody. The diluted sample is added, and any antigen present binds to capture antibody. After a washing step the detecting antibody (biotinylated anti-calretinin antibody) is added and binds to antigen. After another washing step the enzyme conjugate streptavidin-peroxidase is added and binds to detecting antibody. The following substrate TMB / peroxidase reaction is monitored at 450 nm (reference wavelength at 620 nm).

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
13 ml, colour coded yellow, ready for use

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

4.6 **Enzyme Conjugate** **CONJ** 1 vial
13 ml, ready for use
goat anti-rabbit-IgG-peroxidase

4.7	**Wash Buffer** 20 ml, 50x concentrated Dilute content with distilled water to 1 litre total volume	**WASH**	1 via
4.8	**Substrate** 13 ml TMB solution, ready for use	**SUB**	1 vial
4.9	**Stop Solution** 12 ml, ready for use contains 0.3 M sulphuric acid	**STOP**	1 vial
4.10	**Preparation Plate** for dilution	**PLATE**	1 plates
4.11	Adhesive Foil Ready for use	**Foil**	1 piece

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)
- 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 and Samples

Preparation of Reagents

Standards and Controls **CAL 1 – 6** **CON 1 & 2**

Dissolve standards and controls with 200 µl dist. water each, vortex shortly and leave on a roll mixer or similar shaker for minimum 20 minutes. 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.

Wash Buffer WASH

Dilute the content with dist. water to a total volume of 1,000 ml.
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 reaction 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.
Determinations in duplicates are recommended.

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. Incubate for 60 minutes at room temperature on an orbital shaker with medium frequency.

6. Washing: Repeat step 3.

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

8. 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 45 +/- 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.

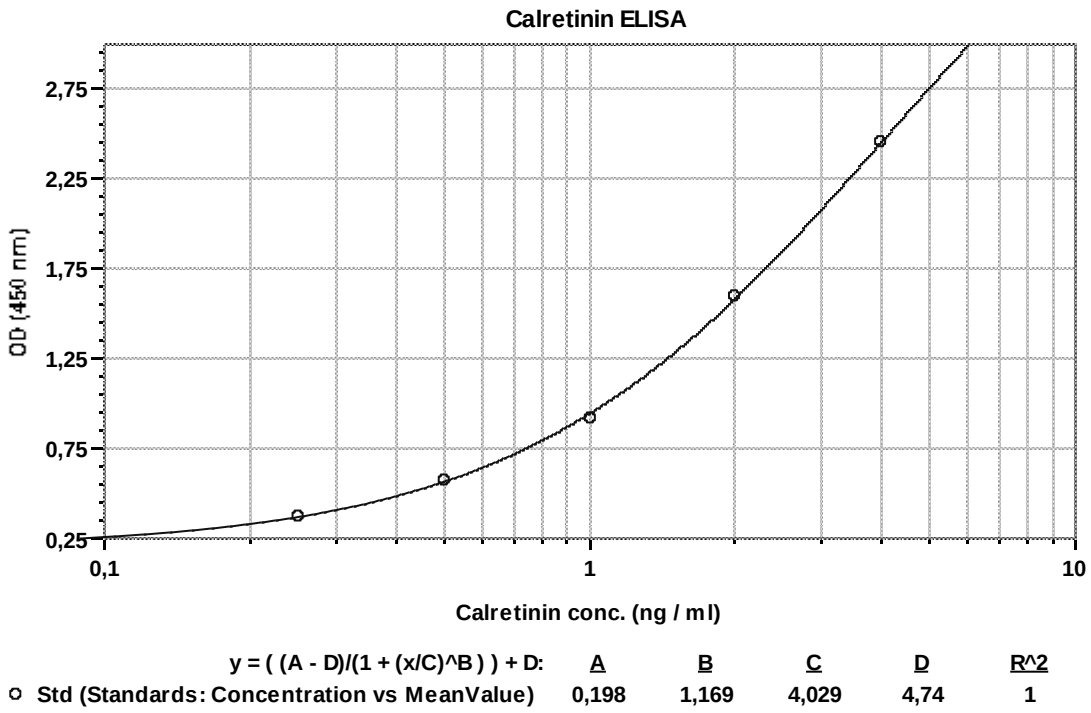
13. Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer within 15 minutes.

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 below 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

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)

11. Symbols used

CONT	Contents		Expiry Date
LOT	Lot Number	+2 +8 °C	Store
	Manufactured by	Σ	Sufficient for
REF	Catalogue Number		Consult Instructions

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
Antiserum	µl	50	50	50
Enzyme Conjugate	µl	100	100	100
Substrate	µl	100	100	100
Stop Solution	µl	100	100	100

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

4 x washing

shake for 6 minutes at room temperature

4 x washing

shake for 60 minutes at room temperature

4 x washing

shake for 15 - 25 minutes at room temperature

reading of absorbance at 450 nm