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Instructions for use

25-OH Vitamin D (total) ELISA

REF **MS E-5800**

25-OH Vitamin D (total) ELISA

1. INTRODUCTION

1.1 Intended Use

The **25-OH Vitamin D (total) ELISA** is an enzyme immunoassay for the quantitative ***in vitro diagnostic*** measurement of total 25-OH Vitamin D (Vitamin D_2 / D_3) in serum or plasma (EDTA, lithium heparin or citrate plasma).

1.2 Summary and Explanation

Vitamin D is a steroid hormone involved in the intestinal absorption of calcium and the regulation of calcium homeostasis. The two major forms of Vitamin D, named Vitamin D_3 (cholecalciferol) and Vitamin D_2 (ergocalciferol), have isomeric structures, but D_2 is supposed to be less active than D_3 [1].
Physiological Vitamin D_3 levels result not only from dietary uptake but can also be produced from a cholesterol precursor, 7-dehydrocholesterol, in the skin during sun exposure. D_2 is obtained from plant sources and only represents less than 5% of the total Vitamin D in the body [2]. In the liver, the Vitamin D is hydroxylated to 25-hydroxyvitamin D (25-OH D), the major circulating metabolite of Vitamin D. Vitamin D and 25-OH D enter the circulation bound to Vitamin D binding protein (VDBP). Upon request, a small portion of 25-OH D is further hydroxylated in the kidney to form the biologically active hormone 1,25 dihydroxyvitamin D (1,25-$(OH)_2$ D) [3]. This process is tightly regulated by the concentration of 1,25-$(OH)_2$ D, parathyroid hormone, hypophosphatemia and ionized calcium levels. Concentrations of 1,25-$(OH)_2$ are about 1000-fold lower than that of 25-OH D [4]. Although 1,25-$(OH)_2$ D portrays the biological active form of Vitamin D, it is widely accepted that the measurement of circulating 25-OH D provides better information with respect to patients Vitamin D status and allows its use in diagnosis of hypovitaminosis [5].
The concentration of 25-OH D decreases during winter time (reduced sun exposure), with dark skin colour and with age [6,7].
Determination of 25-OH D in serum or plasma will support the diagnosis and therapy control of postmenopausal osteoporosis, rickets in children, osteomalacia, renal osteodystrophy, neonatal hypocalcemia and hyperparathyroidism. In addition, the effects of prevailing subclinical Vitamin D deficiency in different European countries is critically discussed [6].
Vitamin D intoxication mostly occurs during a large intake of pharmaceutical preparations of Vitamin D and may lead to hypercalcemia and nephrocalcinosis in susceptible infants.

2. PRINCIPLE OF THE TEST

The 25-OH Vitamin D (total) ELISA is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the **principle of competitive binding**.
The microtiter wells are coated with a monoclonal (mouse) antibody directed towards a unique antigenic site of the 25-OH Vitamin D (25-OH D) molecule.

The patient sample is incubated together with Release Reagent in the wells to dissociate endogenous 25-OH D from Vitamin D binding protein (VDBP). Released 25-OH D then binds to the coated antibody of the well.
After a washing step, biotin-labeled 25-OH D (*Enzyme Conjugate*) and peroxidase-labeled streptavidin (*Enzyme Complex*) are added.
Added Biotin-25-OH D competes with endogenous 25-OH D for the binding to the coated antibody. Bound Biotin – 25-OH Vitamin D is then detected by Streptavidin-HRP.
After incubation, unbound components are washed off.
The amount of bound biotin-streptavidin complex is inversely proportional to the concentration of 25-OH Vitamin D in the sample.
Subsequently, substrate solution is added and the color development is stopped after a defined time.
The intensity of the color formed is inversely proportional to the 25-OH D concentration in the sample. The absorbance is measured at 450 nm with a microtiter plate reader.

3. WARNINGS AND PRECAUTIONS

1. This kit is for in vitro diagnostic use only. For professional use only.
2. All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
3. Before starting the assay, read the instructions completely and carefully. Use the valid version of instructions for use provided with the kit. Be sure that everything is understood.
4. The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch and used in the frame provided.
5. Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
6. Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur.
7. Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.

8. Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
9. Allow the reagents to reach room temperature (21 °C to 26 °C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.
10. Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
11. Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
12. Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
13. Handling should be done in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
14. Do not use reagents beyond expiry date as shown on the kit labels.
15. All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiter plate readers.
16. Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
17. Avoid contact with Stop Solution containing 0.5 M H_2SO_4. It may cause skin irritation and burns.
18. Some reagents contain Proclin 300, BND and/or MIT as preservatives. In case of contact with eyes or skin, flush immediately with water.
19. TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.
20. Chemicals and prepared or used reagents have to be treated as hazardous waste according to the national biohazard safety guideline or regulation.
21. For information on hazardous substances included in the kit please refer to Safety Data Sheets. Safety Data Sheets for this product are available upon request directly from the manufacturer.

4. REAGENTS

4.1 Reagents provided

MS E-5831 **MTP** **96** **Microtiterwells**

Content: 12 x 8 (break apart) strips, 96 wells;
Wells coated with anti-25-OH D antibody (monoclonal).

Standards and **Controls** – ready to use

Cat. no.	Component	Standard	Concentration	Volume/Vial
MS E-5801	**STANDARD A**	Standard A	0 ng/ml	1 ml
MS E-5802	**STANDARD B**	Standard B	5 ng/ml	1 ml
MS E-5803	**STANDARD C**	Standard C	15 ng/ml	1 ml
MS E-5804	**STANDARD D**	Standard D	30 ng/ml	1 ml
MS E-5805	**STANDARD E**	Standard E	60 ng/ml	1 ml
MS E-5806	**STANDARD F**	Standard F	120 ng/ml	1 ml
MS E-5851	**CONTROL 1**	Control 1	For control values and ranges please refer to vial label or QC-Report.	1 ml
MS E-5852	**CONTROL 2**	Control 2		1 ml

Conversion: 1 ng/ml = 2.5 nmol/l

Content: Contain non-mercury preservative.

The standards are calibrated against the following reference material: DEQAS No. 548.

MS E-5826 **RELEASE** **REAG** **Release Reagent** – ready to use

Content: Contains non-mercury preservative.

Volume: 1 x 20 ml

Hazards identification:

H317 May cause an allergic skin reaction.

MS E-5840 **CONJUGATE** **Enzyme Conjugate** – ready to use

Content: 25-OH D antigen conjugated with biotin;
Contains non-mercury preservative.

Volume: 1 x 7 ml

MS E-5841	**COMPLEX**	**Enzyme Complex** – ready to use
Content:	Streptavidin-Peroxidase Conjugate; Contains non-mercury preservative.	
Volume:	1 x 7 ml	

FR E-0055	**SUBSTRATE**	**Substrate Solution** – ready to use
Content:	Tetramethylbenzidine (TMB).	
Volume:	1 x 14 ml	

FR E-0080	**STOP-SOLN**	**Stop Solution** – ready to use
Content:	Contains 0.5 M H_2SO_4. Avoid contact with the stop solution. It may cause skin irritations and burns.	
Volume:	1 x 14 ml	

FR E-0030	**WASH-CONC** **40x**	**Wash Solution** – 40X concentrated
Volume:	1 x 30 ml See „Reagent Preparation".	

Note: Additional *Standard A* for sample dilution is available upon request.

4.2 Materials required but not provided

- A microtiter plate calibrated reader (450 ± 10 nm)
- Calibrated variable precision micropipettes
- Absorbent paper
- Distilled or deionized water
- Incubator 37 °C (98.6 °F)
- Timer
- Graph paper or software for data reduction

4.3 Storage Conditions

When stored at 2 °C – 8 °C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date.
Opened reagents must be stored at 2 °C – 8 °C. Microtiter wells must be stored at 2 °C – 8 °C. Once the foil bag has been opened, care should be taken to close it tightly again.
Opened kits retain activity for 8 weeks if stored as described above.

4.4 Reagent Preparation

Bring all reagents and required number of strips to room temperature prior to use.

Wash Solution
Add deionized water to the 40X concentrated Wash Solution.
Dilute 30 ml of concentrated *Wash Solution* with 1170 ml deionized water to a final volume of 1200 ml.
The diluted Wash Solution is stable for 1 week at room temperature.

4.5 Disposal of the Kit

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Safety Data Sheet, section 13.

4.6 Damaged Test Kits

In case of any severe damage to the test kit or components, the manufacturer has to be informed in writing, at the latest, one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

5. SPECIMEN COLLECTION AND PREPARATION

Serum or plasma (EDTA, lithium heparin or citrate plasma) can be used in this assay.

Do not use haemolytic, icteric or lipaemic specimens.
Please note: Samples containing sodium azide should not be used in the assay.

5.1 Specimen Collection

Serum:
Collect blood by venipuncture (e.g. Sarstedt Monovette for serum), allow to clot, and separate serum by centrifugation at room temperature. Do not centrifuge before complete clotting has occurred. Patients receiving anticoagulant therapy may require increased clotting time.

Plasma:
Whole blood should be collected into centrifuge tubes containing anti-coagulant (e.g. Sarstedt Monovette with the appropriate plasma preparation) and centrifuged immediately after collection.

5.2 Specimen Storage and Preparation

Specimens should be capped and may be stored for up to 7 days at 2 °C – 8 °C prior to assaying.
Specimens held for a longer time (up to 12 months) should be frozen only once at -20 °C prior to assay. Thawed samples should be inverted several times prior to testing.

5.3 Specimen Dilution

If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted with *Standard A* and re-assayed as described in Assay Procedure.
For the calculation of the concentrations this dilution factor has to be taken into account.

Example:
a) dilution 1:10: 10 µl sample + 90 µl *Standard A* (mix thoroughly)
b) dilution 1:100: 10 µl dilution a) 1:10 + 90 µl *Standard A* (mix thoroughly).

6. ASSAY PROCEDURE

6.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.

6.2 Test Procedure

Each run must include a standard curve.

1.	Secure the desired number of microtiter wells in the frame holder.
2.	Dispense **50 µl** of each ***Standard, Control*** and **sample** with new disposable tips into appropriate wells.
3.	Dispense **150 µl *Release Reagent*** into each well. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step.
4.	Incubate for **60 minutes** at **37 °C**.
5.	Briskly shake out the contents of the wells. Rinse the wells **4 times** with **400 µl** diluted *Wash Solution* per well, if a plate washer is used - or - rinse the wells **4 times** with **300 µl** diluted *Wash Solution* per well for manual washing. Strike the wells sharply on absorbent paper to remove residual droplets. **Important note**: The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
6.	Add **50 µl** of ***Enzyme Conjugate*** to each well.
7.	Add **50 µl** of ***Enzyme Complex*** to each well.
8.	Incubate for **30 minutes** at **37 °C**.
9.	Briskly shake out the contents of the wells. Rinse the wells **4 times** with **400 µl** diluted *Wash Solution* per well, if a plate washer is used - or - rinse the wells **4 times** with **300 µl** diluted *Wash Solution* per well for manual washing. Strike the wells sharply on absorbent paper to remove residual droplets.
10.	Add **100 µl** of ***Substrate Solution*** to each well.
11.	Incubate for **15 minutes** at room temperature.
12.	Stop the enzymatic reaction by adding **100 µl** of **Stop Solution** to each well.
13.	Determine the absorbance (OD) of each well at **450 ± 10 nm** with a microtiter plate reader. It is recommended that the wells be read **within 10 minutes** after adding the Stop Solution.

6.3 Calculation of Results

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the Instructions for Use have been calculated automatically using a 4-Parameter curve fit. (4 Parameter Rodbard or 4 Parameter Marquardt are the preferred methods.) Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted or reported as that. For the calculation of the concentrations this dilution factor has to be taken into account.

6.3.1 Example of Typical Standard Curve

The following data is for demonstration only and **cannot** be used in place of data generations at the time of assay.

Standard	Optical Units (450 nm)
Standard A 0 ng/ml	2.10
Standard B 5 ng/ml	1.86
Standard C 15 ng/ml	1.51
Standard D 30 ng/ml	1.10
Standard E 60 ng/ml	0.57
Standard F 120 ng/ml	0.09

7. EXPECTED NORMAL VALUES

It is strongly recommended that each laboratory should determine its own normal and abnormal values.

In a study conducted with apparently healthy individuals (50% white Americans, 20% Hispanics and 30% Afro-Americans), using the 25-OH Vitamin D (total) ELISA the following data were observed:

Population	n	Age (years)	Mean (ng/ml)	Median (ng/ml)	2.5^{th} – 97.5^{th} Percentile (ng/ml)	Range (min. – max.) (ng/ml)
Males	92	10 – 83	21.49	18.66	5.58 – 56.83	5.39 – 58.33
Females	102	15 – 80	22.78	19.56	3.99 – 54.23	3.23 – 65.10
Summer	52	24 – 76	22.20	18.21	5.96 – 57.84	3.74 – 58.33
Winter	60	21 – 66	15.13	13.82	3.71 – 33.45	3.23 – 41.24

It is important for each laboratory to establish its own reference range, representative of its typical population. Factors such as UV exposure, season, race, and dietary intake are all known to affect concentrations of 25-OH Vitamin D in humans. A high prevalence of subclinical 25 OH Vitamin D deficiency has been noted in many countries, particularly in winter months.

The results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests.

A review of the literature suggests the following ranges for the classification of 25-OH Vitamin D status [3]:

Vitamin D status	25-OH Vitamin D (ng/ml)	25-OH Vitamin D (nmol/l)
Deficiency	< 10	< 25
Insufficiency	10 – 29	25 – 72.5
Sufficiency	30 – 100	75 – 250
Toxicity	> 100	> 250

8. QUALITY CONTROL

Good laboratory practice requires that controls be run with each standard curve. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance.
It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels.
The controls and the corresponding results of the QC-Laboratory are stated in the QC-Report added to the kit. The values and ranges stated on the QC sheet always refer to the current kit lot and should be used for direct comparison of the results.
It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results.
Employ appropriate statistical methods for analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid.
In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.
After checking the above mentioned items without finding any error contact your distributor or the manufacturer directly.

9. PERFORMANCE CHARACTERISTICS

9.1 Assay Dynamic Range

The range of the assay is between 3.22 **–** 120 ng/ml.

9.2 Specificity of Antibodies (Cross-Reactivity)

The cross-reactivity is determined according to the Method of Abraham[8].
The following substances were tested for cross-reactivity of the assay:

Substance	Added conc. (ng/ml)	Mean cross-reactivity (%)
25-OH Vitamin D_3	15 – 120	99.50
25-OH Vitamin D_2	1.56 – 12.50	97.43
1,25 (OH)2 Vitamin D_3	12 – 12000	0.98
1,25 (OH)2 Vitamin D_2	12 – 12000	0.16
Vitamin D_3	12 – 12000	13.36
Vitamin D_2	12 – 12000	0.96
3-Epi-25-OH Vitamin D_3	12 – 12000	0.83

9.3 Sensitivity

The Limit of Blank (LoB) is 2.219 ng/ml.
The Limit of Detection (LoD) is 3.224 ng/ml.
The Limit of Quantification (LoQ) is 3.750 ng/ml.

9.4 Reproducibility

9.4.1 Intra Assay

The within assay variability was determined by measuring each sample 10 times per run (n = 10):

Sample	N	Mean (ng/ml)	CV (%)
1	10	15.92	6.3
2	10	30.91	4.0
3	10	80.90	3.1
4	10	106.94	1.5

9.4.2 Inter Assay

The between assay variability was determine by measuring each sample 10 times per run for 3 days (n = 30):

Sample	N	Mean (ng/ml)	CV (%)
1	30	16.87	10.2
2	30	31.26	7.7
3	30	81.49	8.4
4	30	108.83	2.2

9.5 Recovery

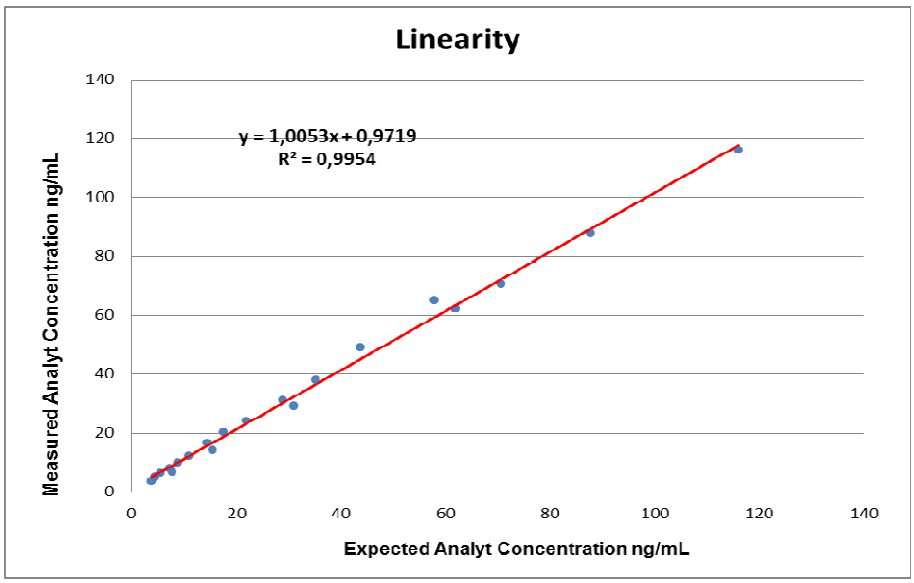
Samples have been spiked by adding 25-OH Vitamin D solutions with known concentrations.
The recovery (%) was calculated by multiplying the ratio of measured and expected values with 100.

		Sample 1	Sample 2	Sample 3	Sample 4
Concentration (ng/ml)		13.30	22.00	40.30	60.00
Average Recovery (%)		98.6	101.6	97.3	106.7
Range of Recovery (%)	**from**	94.6	97.1	89.6	100.0
	to	104.5	104.5	107.7	109.7

9.6 Linearity

Samples were measured undiluted and in serial dilutions from 1:2 to 1:16 with *Standard A*. The recovery (%) was calculated by multiplying the ratio of expected and measured values with 100.

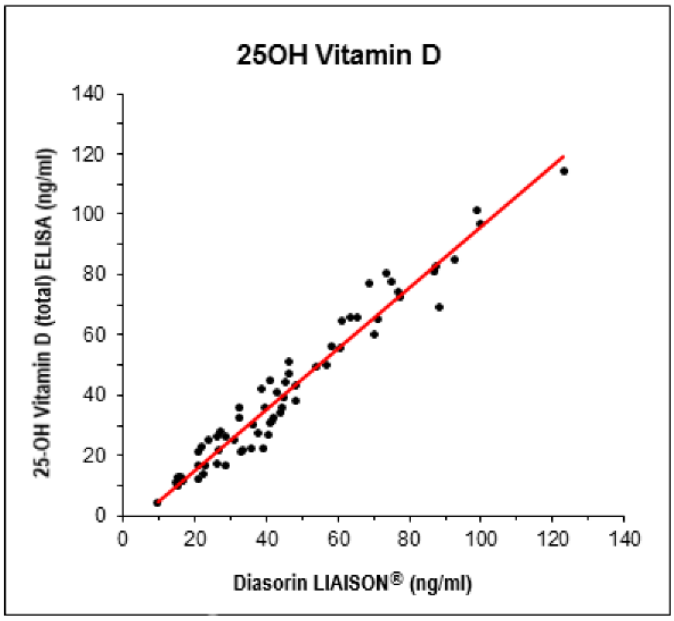
		Sample 1	Sample 2	Sample 3	Sample 4
Concentration (ng/ml)		17.14	62.00	87.70	116.00
Average Recovery (%)		104.0	89.7	111.4	110.8
Range of Recovery (%)	**from**	85.2	85.2	109.5	106.9
	to	111.8	93.5	114.9	113.8



9.7 Comparison Studies

A comparison of 25-OH Vitamin D (total) ELISA (y) and the reference method LIAISON® 25-OH Vitamin D total (Diasorin) (x) using clinical samples gave the following correlation:

n = 67
r = 0.976
y = 1.010x – 4.814



10. LIMITATIONS OF USE

Reliable and reproducible results will be obtained when the assay procedure is performed with a complete understanding of the package insert instruction and with adherence to good laboratory practice.
Any improper handling of samples or modification of this test might influence the results.

10.1 Interfering Substances

Serum, EDTA and citrate plasma:
Haemoglobin (up to 4 mg/ml), bilirubin (up to 0.5 mg/ml) and triglyceride (up to 7.5 mg/ml) have no influence on the assay results.

Li-heparin plasma:
Haemoglobin (up to 0.5 mg/ml), bilirubin (up to 0.5 mg/ml) and triglyceride (up to 7.5 mg/ml) have no influence on the assay results.
Note: Sample concentration will decrease by more than 20% at haemoglobin concentrations > 0.5 mg/ml.

A biotin concentration of up to 1200 ng/ml in a sample has no influence on the assay results.

10.2 Drug Interferences

Until today no substances (drugs) are known to us, which have an influence to the measurement of 25-OH Vitamin D in a sample.

10.3 High-Dose-Hook Effect

A High-Dose-Hook Effect is not known for competitive assays.

11. LEGAL ASPECTS

11.1 Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.
The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact the manufacturer.

11.2 Therapeutic Consequences

Therapeutic consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 11.1. Any laboratory result is only a part of the total clinical picture of a patient.

Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutic consequences be derived.
The test result itself should never be the sole determinant for deriving any therapeutic consequences.

11.3 Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement.
Claims submitted due to customer misinterpretation of laboratory results subject to point 11.2 are also invalid. Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

12. REFERENCES / LITERATURE

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Symbols:

	Storage temperature		Manufacturer		Contains sufficient for <n> tests
	Use-by date		Batch code		For in-vitro diagnostic use only!
	Consult instructions for use		Content		CE marking of conformity
	Caution		Catalogue number		Distributor
	Date of manufacture				