FluoBolt™-Vitamin D

METAL-ENHANCED FLUORESCENCE IMMUNOASSAY FOR THE QUANTITATIVE DETERMINATION OF VITAMIN D IN HUMAN SERUM AND DRIED CAPILLARY BLOOD

Cat. Code. FIA-1709-C5-R
96 Well Format

FOR RESEARCH ONLY
NOT FOR USE IN DAGNOSTIC PROCEDURES

FIANOSTICS
Fluorescence Immunoassays

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CONTENT

1) INTENDED USE

The FluoBolt™-Vitamin D Kit is a metal enhanced fluorescence immunoassay (MEF-FIA) for the quantitative determination of 25-hydroxyvitamin D3 [25(OH)D3] in human serum and dried capillary blood. The determined value is used to observe the vitamin D status and can indicate a possible insufficiency or excess.

The application of the test is limited to certified laboratories that meet the requirements to perform tests of medium or high complexity.

The vitamin D status in blood is highly variable and depends on factors such as season, time of day, exposure to sunlight, use of sunscreen and age.

2) SUMMARY AND EXPLANATION

Vitamin D is formed in the skin by exposure to UV light (~90%) or to a lesser extent ingested with food (~10%). The two main metabolites are vitamin D3 (cholecalciferol), which is mainly formed in the skin or absorbed through animal products, and vitamin D2 (ergocalciferol), which is mainly found in plant foods.

Circulating vitamin D2 and D3 are converted to 25(OH)D in the liver and subsequently to active form 1,25(OH)2D in the kidneys. In the blood, vitamin D metabolites are mainly bound to the vitamin D binding protein (VDBP), less than 1% circulates in free form. Due to its low biological activity and longer half-life, 25(OH)D is considered a storage form and is a good indicator of vitamin D status in the blood.

Vitamin D has important functions in bone and mineral metabolism, as well as for the immune and cardiovascular systems, and general well-being. A deficiency (hypovitaminosis D) with a serum concentration ≤20 ng/ml (50 nmol/l) is common worldwide and is caused by insufficient intake or too little exposure to sunlight. It is generally estimated that ~50% of the population is inadequately supplied. Low vitamin D levels can promote fatigue, chronic deficiency causes rickets in children and osteomalacia in adults and can be treated by vitamin D intake.

Regular monitoring of vitamin D status, especially in people at increased risk of deficiency, can enable early detection and help prevent consequential health damage.

Test Principle:

The FluoBolt™-Vitamin D Kit is a competitive fluorescence immunoassay based on the FluoBolt™-platform (FIA-1700). The 25(OH)D3 present in the patient sample (serum or dried capillary blood) competes with biotin-labeled 25(OH)D3 for the binding sites of anti-25(OH)D3 antibodies coated on the Metal Enhanced Fluorescence microplate (MEF-MTP). The amount of fluorescence units (FUs) measured after addition of a fluorescently labeled streptavidin solution with a fluorescence microplate reader is indirectly proportional to the 25(OH)D3 concentration in the sample. Calibrators with a known amount of 25(OH)D3 are used to create calibration curves and thus quantify the concentration of an unknown sample.

3) CONTENTS OF THE KIT

ID	KIT COMPONENTS	Quantity
MIX	Transparent microplate for standard and sample preparation, packed in plastic bag	1x 96 well
PM	Black MEF-MTP pre-coated with anti-25(OH)D3 antibody; vacuum-packed in an aluminum bag	1x 96 well
PA5	Detection solution consisting of biotin-labeled 25(OH)D3 and fluorescently labeled streptavidin in buffer with release reagent; in dark glass vial with screw cap, lyophilized	1x 5 ml
PS	Standards 1-6 consisting of 25(OH)D3 in human serum (200, 100, 50, 25, 12.5, 0 ng/ml), in glass vials with white screw cap, lyophilized	6x 0.1 ml
PCA/B	Control A (high, yellow screw cap) and B (low, green screw cap) in glass vials, lyophilized; the target values are stated on the label.	2x 0.1 ml
PR	Sample release reagent, in plastic bottle with natural cap, powder	2x 104 mg
PD	Dilution buffer, in plastic bottle with natural cap, ready to use	1x 40 ml
WP	Wash buffer concentrate 20x, in plastic bottle with natural cap	1x 25 ml

4) ADDITIONAL MATERIAL SUPPLIED WITH THE KIT

- 1 self-adhesive plastic film
- Quality control data sheet
- Protocol sheet
- Instruction for use
- Desiccant bag for plate storage

5) MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- Precision pipettes calibrated to 10µl, 20µl, 50µl, 200µl, 500µl and disposable tips
- Plate washer, multi-channel pipette or multi-dispenser pipette for washing
- Refrigerator with 4°C (2-8°C)
- Fluorescence microplate reader
- Graph paper or software to calculate the results.

In addition, when using dried capillary blood samples:

- Microplate for sample extraction
- Microplate shaker

6) REAGENT AND SAMPLE PREPARATION

All reagents of the kit are stable at 4°C (2-8°C) until the expiration date stated on the label of each reagent.

Sample collection

1) **Serum samples**
   Collect venous blood samples by using standardized blood collection tubes for serum. We recommend serum separation by centrifugation as soon as possible, e.g. 10 min at 2000 x g, preferably at 4°C (2-8°C). The obtained serum samples should be measured as soon as possible. For longer storage, samples should be aliquoted and stored at temperatures of -20°C or below. Freeze and thaw samples no more than 4 times. Lipemic or hemolytic samples may give erroneous results. Samples should be mixed well before analysis. For more information on sample stability, contact us by e-mail at support@fianostics.at or by phone at +43/2622/27514.

2) **Capillary blood samples**
This assay has also been validated for the use of dried capillary blood samples collected with 20 µl Mitra® microsamplers (Neoteryx LLC, Torrence CA 90501, USA). Mitra samples have to be extracted with sample release buffer (PR) prior to use in the assay (see "7.2 Assay Procedure"). Details on the collection procedure can be found in the manual of our separately available blood collection kit (Cat. No. INO-2201B).

Reagent preparation:

1) **Preparation of the wash buffer**
Bring the 20x wash buffer concentrate (WP) to room temperature. Make sure that the solution is clear and without any salt precipitates before further diluting the concentrate. Dilute the WP to the working concentration (1x) prior to use in the assay by adding the appropriate amount of distilled or deionized water (dH_2O), e.g., 25 ml WP + 475 ml water. Undiluted WP can be stored at 4°C (2-8°C) until the expiry date printed on the label. Diluted WP can be stored at 4°C (2-8°C) for up to one month. Use only diluted WP in the assay.

2) **Preparation of standards and controls**
Reconstitute the standards and controls by adding 100µl dH_2O to the freeze-dried solid at the bottom of the vials. Check that the solid is indeed at the bottom and not elsewhere in the vial (e.g. on the sides) before adding dH_2O. Close the vials and leave them at room temperature (18-26°C) for 10 minutes. Then homogenize briefly with e.g. a vortex mixer. Reconstituted standards/controls can undergo up to five freeze-thaw cycles.

3) **Preparation of the detection solution**
Reconstitute the detection solution by adding 5 ml of sample dilution buffer (PD) to the freeze-dried solid at the bottom of the vial. Follow the same procedure as described in point 2). The reconstituted detection solution can be frozen and thawed up to 4 times.

4) **Preparation of sample release (PR) buffer solution when using capillary blood samples on Mitra® microsamplers.**
Dissolve the sample release reagent (PR) by adding 12 ml of dilution buffer (PD) to the solid in the vial. Follow the same procedure as described in point 2). The powder must be completely dissolved before use. The PR buffer solution has a shelf life of up to 7 days at 4°C (2-8°C).

7) ASSAY PROCEDURE

All reagents and samples must be brought to room temperature (18-26°C) prior to use in the assay.

1) **Generating the plate-layout**
Enter the positions for standards, controls and samples on the protocol sheet. We generally recommend running standards and samples in duplicates.

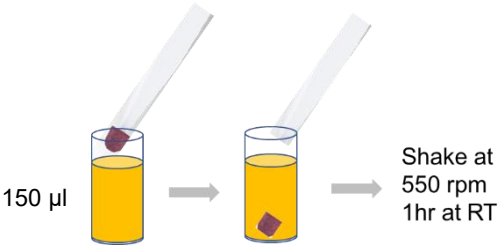
2) If you are using **serum samples**, please proceed to step 4.

3) If you are using dried capillary blood samples on **Mitra® microsamplers,** proceed as follows**:**
- Pipette 150 µl of the prepared **PR buffer solution** for each collected sample into suitable vials (1-1.5 ml) or into a microplate.

- Add the adsorbent pad of the 20 µl Mitra® microsampler of each sample by gently stripping it off at the edge of the vial or well of the microplate**. Be careful not to touch the adsorptive pad** of the Mitra® microsampler.

- Shake for 1 hour at room temperature using a suitable microplate shaker at 550 rpm.

Procced with step 4.

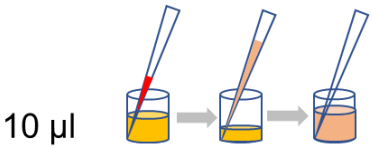


4) Remove the **transparent microplate (MIX)** used for pre-mixing standards, controls or samples with detection solution from the plastic bag.

Add 50 µl **detection solution (PA5)** into each required well of the MIX plate.

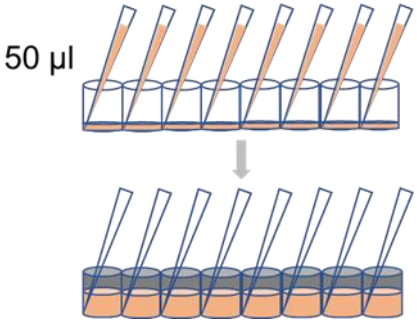
5) Now add 10 µl of standard, control, or sample to the wells of the MIX plate according to the marked positions on the protocol sheet.
It is important to ensure that the pipette tip is guided along the edge to the bottom of the well to ensure adequate mixing with the detection solution.

Use a fresh pipette tip for each well.



6) Remove **the black MEF-microplate (PM)** from the aluminum bag. Seal all wells that will not be used in the following assay run with the supplied self-adhesive plastic film (cut to fit).

7) Now transfer 50µl of the pre-mixed standards, controls, and samples from the transparent MIX plate to the black PM plate with an 8-channel pipette. Again, guide the pipette tips along the edge to the bottom of the well before emptying the tips.



8) When all wells intended for use are filled, close the wells thoroughly with the supplied self-adhesive film and incubate for 60 minutes in the dark at room temperature (18-26°C).

9) Remove the contents of the wells by discarding or aspirating and wash the plate 3x with 200 µl diluted WP per well. After the final washing step, remove the remaining liquid by vigorously tapping the plate with the wells down against a stack of paper towels or similar absorbent material.

10) Read the empty plate immediately or no more than 5 minutes after washing with a microplate reader with excitation/emission wavelengths suitable for the fluorescent dye used (the Ex/Em maxima for FITC: 495/518 nm, Cy3: 550/570 nm, Cy5: 650/670 nm and AlexaFluor680: 679/702 nm).

    The sensitivity setting of the reader (gain) should be selected so that a difference of at least 10 000 FUs between the 0 ng/ml and the 200 ng/ml standard is achieved.

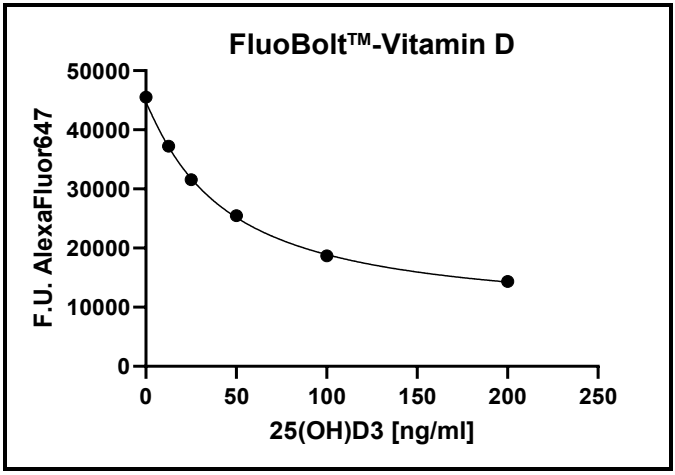
11) Samples that exceed the highest standard signal must be retested after dilution with the supplied sample dilution buffer (PD).

8) CALCULATION AND INTERPRETATION OF RESULTS

Calculation

Create a calibration curve from the FUs of the standards using commercially available software or graph paper. Read concentrations of the controls and samples from this calibration curve. The assay was validated using a 4PL algorithm. Other curve fitting methods must be evaluated by the user.

Example of typical calibration curve:



The quality control (QC) protocol supplied with the kit displays the results of the final release QC for each kit lot at the production date. The fluorescence intensity received by the customer may differ due to various influences and/or due to the normal decrease of signal intensity during shelf life.

However, this does not affect the validity of results if the supplied kit controls are within the specifications (target ranges: see labels).

The concentrations read from the respective calibration curve yield the concentrations of 25(OH)D3 in ng/ml.
Conversion factor: 25-OH-vitamin D [ng/ml] x 2.5 = nmol/l

Values obtained with capillary dried blood samples must be converted to serum equivalent concentrations using the following formula:

Serum concentration = 2,5811 x capillary blood conc. + 11,484

Interpretation

<10 ng/ml	severe deficiency
10-30 ng/ml	insufficiency
30-50 ng/ml	adequate supply
50-100 ng/ml	increased level (substitution?)
>100 ng/ml	overdose (toxicity)

The specified concentration ranges for classifying the vitamin D supply status should be considered as a recommendation. Factors such as diet, season, skin tone, and age influence normal 25(OH)D levels.

The results of this test should always be interpreted in conjunction with the individual's medical history, clinical presentation, and other findings.

9) PERFORMANCE CHARACTERISTICS

Analytical specificity:

The assay detects 25(OH)D3 and 3-epi-25(OH)D3. There is no cross-reactivity with 25(OH)D2.

Metrological traceability

The following international reference samples were tested in the assay:

Quantimetrix Complete D® 25-OH Vitamin D Control (catalog#1290-01), traceable to NIST standard SRM 972a.

Results:

	25(OH)D3 [ng/ml]	
	Target Values (LC-MS/MS)	**FluoBolt™ Vitamin D**
Level 1	9	6
Level 2	29	20

Precision:

Intra-assay precision: 4 samples with known concentrations were tested 3 times within 1 assay run.

Inter-assay precision: 4 samples with known concentrations were tested in duplicates in 3 different assay runs.

Inter-lot precision: 3 samples with known concentrations were tested in duplicates with 3 different kit lots.

Intra-assay (n=3)	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Mean [ng/ml]	24,7	34,6	57,6	69,4
SD [ng/ml]	2,6	2,7	4,0	7,5
CV (%)	10,3%	7,7%	7,0%	10,7%
Inter-assay (n=3)	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Mean [ng/ml]	45,7	28,8	20,9	42,0
SD [ng/ml]	4,1	2,8	0,8	2,4
CV (%)	9,0%	9,7%	3,6%	5,7%
Inter-lot (n=3)	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Mean [ng/ml]	42	23	17	134
SD [ng/ml]	2,5	3,9	0,5	11,5
CV (%)	6%	17%	3%	9%

Detection limit:
Lower Limit of Detection (LoD):
The LoD corresponds to the lowest concentration of 25(OH)D3 that can be detected. The LoD of this test was determined according to the concentration resulting from the mean value of the 0 ng/ml calibrator plus three standard deviations in 6 assay runs performed on different days and is estimated to be 9.8 ng/ml.
Lower Limit of Quantification (LLoQ): 12.5 ng/ml

Linearity: For a linearity study, three different serum samples were measured within the concentration range of the standards and serially diluted with standard 6. The mean linearity of dilution was 121%.

Clinical agreement: For a method comparison with a competitor's automated immunoassay, 48 human serum samples were measured with the FluoBolt™-Vitamin D assay and the readings compared:
Pearson r = 0.9384 (***p<0.0001).

10) LIMITATIONS & TECHNICAL HINTS

Limitations:
The following information refers to limitations of the assay:

- The use of the FluoBolt™-Vitamin D assay is limited to trained laboratory personnel. Not for home use.

- Performance characteristics of this assay have not been determined in conjunction with assays from other manufacturers for vitamin D. Laboratories are responsible for establishing their own performance characteristics.

- Results obtained with the assay must not be used interchangeably with values obtained with the test methods of other manufacturers.

- Performance was established only with sample types listed in the intended use. Other sample types have not been evaluated and should not be used with this assay.

- Results are not intended as a basis for patient management decisions.

Technical hints:

- Do not mix or replace reagents with those of other lots or sources.

- Do not mix caps of different reagents and do not use reagents between lots.

- Do not use reagents beyond the expiry date.

- Protect reagents from direct sunlight.

- To ensure accurate results, pay attention to proper adhesion of the self-adhesive films for a complete seal of the wells during incubation steps.

- Avoid foaming when mixing reagents.

11) PRECAUTIONS

- Liquid reagents contain ≤0.1% Proclin 300 as a preservative. Proclin 300 is not toxic in the concentrations used in this kit. It may cause allergic skin reactions – avoid contact with skin, eyes and mucous membranes.

- Do not pipette by mouth.

- Do not eat, drink, smoke or apply cosmetics where reagents are used.

- Wear gloves, protective glasses and a lab coat when performing this assay.

- Chemicals and prepared, used, unused or expired reagents must be disposed of as hazardous waste in compliance with the respective national regulations.

- All serious adverse events relating to this device shall be reported to the manufacturer and the competent authority of the member state where the user and/or patient is established.

- All reagents in this kit containing human serum where tested negative for anti-HCV, HBsAg and antibodies against HIV 1 and HCV RNA virus, respectively. Nevertheless, the presence of such infectious agents cannot be ruled out with absolute certainty. The reagents should therefore be treated as potentially infectious material.

12) LITERATURE

Vitamin D status in healthy populations worldwide: a systematic review protocol. Dunlop E et al., JBI Evid Synth. 2023;10.11124/JBIES-22-00354. doi:10.11124/JBIES-22-

Prevalence of vitamin D deficiency and associated risk of all-cause and cause-specific mortality among middle-aged and older adults in the United States. Wang TY et al., Front Nutr. 2023;10:1163737. doi:10.3389/fnut.2023.1163737

Vitamin D testing and treatment: a narrative review of current evidence. Pilz S et al., Endocr Connect. 2019 Feb 1;8(2):R27-R43. doi: 10.1530/EC-18-0432.

Vitamin D Testing-Where Are We and What Is On The Horizon? Heureux N. Adv Clin Chem. 2017;78:59-101. doi:10.1016/bs.acc.2016.07.002

Relative Efficacy of Vitamin D2 and Vitamin D3 in Improving Vitamin D Status: Systematic Review and Meta-Analysis. Balachandar R et al., Nutrients. 2021 Sep 23;13(10):3328. doi: 10.3390/nu13103328

Vitamin D Standardization Program (VDSP). Standardizing vitamin D assays: the way forward. Binkley N, Sempos CT. J Bone Miner Res. 2014;29(8):1709-1714. doi:10.1002/jbmr.2252

An assessment of 25-hydroxyvitamin D measurements in comparability studies conducted by the Vitamin D Metabolites Quality Assurance Program. Bedner M, Lippa KA, Tai SS. Clin Chim Acta. 2013 Nov 15;426:6-11. doi: 10.1016/j.cca.2013.08.012.

13) NOTES

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