FastPack® Vitamin D Immunoassay

For the Quantitative Measurement of 25-hydroxyvitamin D and other hydroxylated metabolites in Human Serum and Plasma

The concentration of 25-hydroxyvitamin D in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity. The results reported by the laboratory to the physician must include the identity of the 25-hydroxyvitamin D assay method used. Values obtained with different assay methods should not be used interchangeably.

INTENDED USE

The FastPack® Vitamin D Immunoassay is intended for the quantitative determination of total 25-hydroxyvitamin D and other hydroxylated metabolites in human serum and plasma. The assay is to be used as an aid in the assessment of vitamin D sufficiency in adults. The FastPack® Vitamin D Immunoassay is intended for use with the FastPack® and FastPack® IP Analyzers.

SUMMARY

Vitamin D is a commonly used term for a family of closely related seco-steroids. It is a fat-soluble steroid prohormone mainly produced photochemically in the skin from 7-dehydrocholesterol.[1,2] Upon exposure to sunlight, 7-dehydrocholesterol, located deep in the actively growing layers of the epidermis, undergoes photolytic cleavage of the "B" ring to yield pre-vitamin D_3 which is isomerized to vitamin D_3 (cholecalciferol).[3] Two forms of vitamin D are biologically relevant – vitamin D_3 (Cholecalciferol) and vitamin D_2 (Ergocalciferol). Both vitamins D_3 and D_2 can be absorbed from food, with vitamin D_2 being an artificial source, but only an estimated 10-20% of vitamin D is supplied through nutritional intake.[1] Vitamins D_3 and D_2 can be found in vitamin supplements. Vitamin D is converted to the active hormone 1,25-(OH)2-vitamin D (Calcitriol) through two hydroxylation reactions. The first hydroxylation converts vitamin D into 25-OH vitamin D and occurs in the liver. The second hydroxylation converts 25-OH vitamin D into the biologically active 1,25-(OH)2-vitamin D and occurs in the kidneys as well as in many other cells of the body. Most cells express the vitamin D receptor and about 3% of the human genome is directly or indirectly regulated by the vitamin D endocrine system.[1]

Vitamin D is stored in adipose tissue and enters the circulation bound to vitamin D binding protein (VDBP) and albumin. The major storage form of vitamin D is 25-OH vitamin D and is present in the blood at up to 1,000 fold higher concentration compared to the active 1,25-(OH)2-vitamin D. 25-OH vitamin D has a half-life of 2-3 weeks versus 4 hours for 1,25-(OH)2-vitamin D. Therefore, 25-OH vitamin D is the analyte of choice for determination of the vitamin D status.[4,5] Serum concentration of 25-OH vitamin D is considered to be the most reliable measure of overall vitamin D status and thus can be used to determine if a patient is vitamin D sufficient.[2] Assessment of vitamin D status may also be required to determine the cause of abnormal serum calcium concentrations in patients.

Epidemiological studies have shown a high global prevalence of vitamin D insufficiency and deficiency.[8] The measurement of vitamin D status provides opportunities for preventive and therapeutic interventions.[7,8,9] Vitamin D deficiency is a cause of secondary hyperparathyroidism and diseases resulting in impaired bone metabolism (like rickets, osteoporosis, osteomalacia).[4,10,11] Reduced 25-OH vitamin D concentrations in blood (vitamin D insufficiency) have been associated with an increasing risk of many chronic diseases, including common cancers, autoimmune or infectious diseases or cardiovascular problems.[1,4,8,10,12-14]

TEST PRINCIPLE

The FastPack® Vitamin D Immunoassay is a paramagnetic particle chemiluminescence immunoassay based on the "competitive" principle.

- Endogenous Vitamin D in the sample will be mixed with pretreatment buffer then added into pack
- Primary incubation: A monoclonal (mouse) anti-vitamin D antibody labeled with alkaline phosphatase [100 µL] reacts with vitamin D from the pre-treated patient sample, control or calibrator [100 µL].
- Secondary incubation: Vitamin D covalently coupled to biotin and pre-bound to streptavidin-coated paramagnetic particles [150 µL] is combined with the immunoreactant complex. Conjugate not reacted with the sample will bind to the unoccupied binding sites of the vitamin D–biotin-streptavidin coated paramagnetic particles.
- Removal of unbound materials: The paramagnetic particles are repeatedly washed with wash buffer [0.2 mL/wash] to remove unbound materials.
- Substrate addition and detection: Chemiluminogenic substrate [140 µL] is added to the solid-phase bound complex and results in "glow" chemiluminescence, which is measured using the FastPack® System.
- The amount of bound labeled-antibody is inversely proportional to the concentration of vitamin D in the sample.

REAGENTS – Content and Concentration

Each 30-Test FastPack® Vitamin D Immunoassay Kit ***contains:***

- 30 FastPack Vitamin D Reagent Packs (IP Pipette Format)
- 32 FastPack Vitamin D Pretreatment Vials

Each 50-Test FastPack® Vitamin D Immunoassay Kit ***contains:***

- 50 FastPack Vitamin D Reagent Packs
- 52 FastPack Vitamin D Pretreatment Vials

Each Pretreatment Vial contains:

- Vitamin D Pretreatment Buffer, 200 µL
  Perfluorooctanoic acid (PFOA) in purified water containing 0.1% ProClin 300 as a preservative.

Each FastPack Vitamin D Reagent Pack contains:

- Paramagnetic Particles, 150 µL
  Biotin-25-OH vitamin D bound to streptavidin-coated paramagnetic particles in buffer containing 0.1% ProClin 300 as a preservative.
- Vitamin D Antibody Solution, 100 µL
  Antibody solution containing a mouse monoclonal anti 25-OH vitamin D antibody labeled with alkaline phosphatase in a protein matrix.
- Wash Buffer, 2.0 mL
  Tris buffer containing surfactants.
- Substrate, 140 µL
  ImmuGlow™ Plus: Indoxyl-3-phosphate and lucigenin in buffer containing preservatives.

Materials required but not provided

- FastPack® or FastPack® IP System
- FastPack® Vitamin D Calibrator Kit – Cat. No. 25000062
- FastPack® Vitamin D Control Kit – Cat. No. 25000061

WARNINGS AND PRECAUTIONS

- For *in-vitro* diagnostic use only.
- Do not pipette by mouth.
- Do not eat, drink or smoke in designated work areas.
- Wash hands thoroughly after handling specimen.
- HAMA Interference: some individuals have antibodies to mouse protein (HAMA), which can cause interference in immunoassays that employ antibodies derived from mice[6].
- FastPack® reagents are stable until the expiration date on the label when stored and handled as directed. Do not use FastPack® reagents beyond the expiration date.
- Discard used FastPacks into a Biohazard container.
- Proclin 300 is an irritant. The following are appropriate Risk (R) and Safety (S) phrases for Proclin 300:
  R43 May cause sensitization by skin contact
  S28-37 After contact with skin, wash immediately with plenty of soap and water. Wear suitable gloves.

STORAGE INSTRUCTIONS

Store at 2 – 8 °C.

SPECIMEN COLLECTION/PREPARATION

1. Serum, EDTA or lithium-heparin plasma samples can be used for the FastPack® Vitamin D Immunoassay.
2. The National Committee for Clinical Laboratory Standards (NCCLS) provides the following recommendations for handling, processing and storing blood.[7,8, 15]
   A. Collect all blood samples observing routine precautions for venipuncture.
   B. For serum samples:
      a. Serum should be separated from the cells by centrifugation within 3 hours from time of collection and stored at 2-8 ºC. Transfer the serum from the original tube for storage.
      b. If not tested within 24 hours, the sample should be frozen at –20 ºC or colder.[15]
   C. For plasma samples:
      a. Collect samples in an EDTA (lavender top) or heparinized (green top) tube.
      b. Mix the tube immediately after collection by gently inverting it several times.
      c. Plasma should be separated from the cells by centrifugation within 3 hours from time of collection and stored at 2-8 ºC. Transfer the plasma from the original tube for storage.
      d. If not tested within 24 hours, the sample should be frozen at –20 ºC or colder.[15]
   D. Samples should be free of red blood cells, or other particulate material for optimal results.
   E. Samples showing particulate matter should be centrifuged prior to use.

F. Samples showing turbidity (high lipid content) should not be used.
G. Ensure the samples are free of bubbles.
H. All calibrators, controls, and serum samples need to be pre-treated by diluting 1:3 with the Vitamin D Pretreatment Buffer (1 part sample, 2 parts pretreatment buffer), per the ASSAY PROCEDURE below.

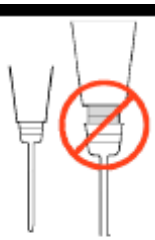
ASSAY PROCEDURE

See the QA Manual or the FastPack® System Procedure Manual for detailed instructions for running FastPack® assays.

1. Write the Patient's Name/ID # and the operator's initials on the FastPack® label.

2. Press and hold the pipette plunger down completely so that the metal grippers are extended and open, as shown in Figure 1.

3. While holding the plunger down, firmly press the pipette into the pipette tip until it snaps in place, then release the plunger.



4. Be sure the pipette tip is seated properly on the end of the pipette, as seen in Figure 2.

5. Verify that the pipette tip is properly seated by gently pressing the plunger down to the first stop and releasing. An audible "Click" may occur if the piston is not seated properly.

6. Gently press the pipette plunger down to the first stop and hold. Place the pipette tip into the sample tube; withdraw sample by slowly releasing the plunger. Inspect the pipette tip to confirm there are no air bubbles in the sample.

7. All samples must be pretreated by diluting 1:3 with FastPack® Vitamin D Pretreatment Buffer (1 part sample, 2 parts pretreatment buffer). Eject sample into the tube (pre-loaded with 200 µL Pretreatment Buffer) by pressing down on the pipette plunger to the first stop. Replace the screw cap tightly onto the buffer tube.

8. Invert the buffer tube at least 3 times to thoroughly mix together the sample and buffer. The act of mixing the sample and buffer releases the vitamin D present, therefore making it available for the assay.

9. Withdraw the sample from the pre-treatment buffer tube using the same pipette tip as before and following the same technique as in Step 6 above.

10. With your finger off the pipette plunger, fully insert the filled pipette tip into the FastPack® Injection Port. It should fit tightly. Refer to Figure 3.

11. Be sure the pipette tip is seated properly into the injection port. Refer to Figures 4 and 5.

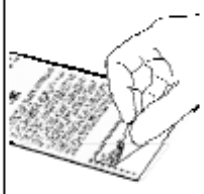
12. In one continuous motion, quickly press the pipette plunger all the way down. This action will simultaneously inject the sample into the FastPack® and automatically eject the pipette tip.

13. Be certain not to unseat the pipette tip from the injection port. The tip acts as a plug to seal the sample in the FastPack®. If you observe a leak, properly dispose of the pack and begin the procedure with a new FastPack®.

14. Place the FastPack® on the door of the analyzer. Align the pins on the door with the holes in the FastPack®. Close the analyzer door.

15. Press the blue Start button on the analyzer.

16. Open the analyzer door when the test is complete. Remove the FastPack® and print the results on the attached label.



17. Peel off the label and place it in the patient record.

SUPPORT

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INSTRUMENTATION
FastPack® or FastPack® IP System

DETAILS OF CALIBRATION
During the FastPack production process, Qualigen generates a master standard curve and places this information in the barcode of each FastPack® label, where it can be read by the FastPack Analyzer during the testing sequence. The FastPack Analyzer must be calibrated by the user to ensure that it is properly adjusted for the particular lot of FastPacks that is being used. Separate calibrations must be run for each type of test, i.e. Total PSA, Testosterone, Vitamin D, TSH, free T4, etc. The frequency of calibration varies for each test type. For the FastPack® Vitamin D Immunoassay, the FastPack® Analyzer must be calibrated once every 30 days or whenever a new lot of Vitamin D FastPacks are to be used.

Whenever the user performs an initial calibration for a particular lot of FastPacks or uses a new lot of calibrator, 2 FastPacks must be run for calibration (duplicates). Whenever recalibration is performed with the same lot of FastPacks and calibrator, 2 FastPacks must be run for calibration. See FastPack® System Procedure Manual for "Running a Calibration".

Use FastPack® Vitamin D Calibrator Kit – Cat. No. 25000062

RESULTS
The FastPack® Analyzer uses the information from the barcode to construct a lookup table of x,y values that represent the standard curve and estimates the concentration of unknown samples by linear interpolation.

QUALITY CONTROL
Quality control materials simulate real specimens and are essential for monitoring the system performance of assays. Good Laboratory Practices (GLP) include the use of control specimens to ensure that all reagents and protocols are performing properly. See FastPack® System Procedure Manual for "Control Testing". At least two levels of quality control materials should be used.

Users should follow the appropriate federal, state and local guidelines concerning the running of external quality controls.

Controls available: FastPack® Vitamin D Control Kit – Cat. No. 25000061

LIMITATION OF PROCEDURE
- Plasma samples to be collected using lithium-heparin or EDTA as the anticoagulants.
- Do not use lipemic samples because lipemic samples will generate a falsely low result.
- Specimens can be measured within the reportable range of the limit of quantitation (12.9 ng/mL) and the upper end of the calibration range, 150 ng/mL.
- Samples >150 ng/mL should be reported as such or re-run using another method.
- Specimen from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibodies (HAMA). Such specimens may show either falsely elevated or depressed values when tested with assay kits employing mouse monoclonal antibodies.
- Heterophilic antibodies in a sample have the potential to cause interference in immunoassay systems. Infrequently, vitamin D levels may appear depressed due to heterophilic antibodies present in the patient's sample or to nonspecific protein binding. If the vitamin D level is inconsistent with clinical evidence, additional vitamin D testing is suggested to confirm the result.
- For diagnostic purposes, the FastPack® Vitamin D Immunoassay should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.
- The FastPack® Vitamin D Immunoassay has not been evaluated in Point of Care settings.

EXPECTED VALUES/REFERENCE INTERVALS

Each laboratory should determine ranges for their local population. There is no universal agreement on the optimal concentration of Vitamin D. Ranges should be based on clinical decision values that apply to both sexes rather than population based reference ranges. A reference interval study employing serum samples from 367 subjects representing 5 different geographic regions of the United States with sampling taking place in Winter, Spring, and early Summer months yielded the results in the table below. The non-parametric 2.5^{th} - 97.5^{th} percentile of 13.7 - 57.3 ng/mL provides the reference interval determined from this study.

Observed values	
Mean	27.6 ng/mL
Median	24.2 ng/mL
2.5^{th} - 97.5^{th} percentile	13.7 - 57.3 ng/mL

SPECIFIC PERFORMANCE CHARACTERISTICS

Precision

Precision was evaluated following the CLSI EP5-A2 guidance. Four samples with concentrations of ~25, ~30, ~45, and ~80 ng/mL were tested in duplicate determinations in each of two runs per day on each of two FastPack® Analyzers, each paired with an individual FastPack Reagent lot over a period of 20 days to yield 160 replicate determinations of each sample (80 replicates on each of two analyzers). Within-run, between-run, and between-day components of variation were calculated as well as total imprecision using a fully nested 2-way random factor ANOVA model with runs nested within days. The tables below present the results by instrument/reagent combination:

Analyzer 1, Reagent Lot 1

		Within-Run		Between-Run		Between-Day		Total	
	Average	SD	% CV	SD	% CV	SD	% CV	SD	% CV
Sample 1	27.3	2.8	10.2	1.3	4.9	1.9	7.1	3.7	13.4
Sample 2	31.1	3.3	10.7	0.0	0.0	1.8	5.7	3.8	12.1
Sample 3	45.5	3.9	8.5	0.0	0.0	2.0	4.3	4.3	9.5
Sample 4	84.9	4.1	4.8	0.0	0.0	3.2	3.7	5.1	6.1

Analyzer 2, Reagent Lot 2

		Within-Run		Between-Run		Between-Day		Total	
	Average	SD	% CV	SD	% CV	SD	% CV	SD	% CV
Sample 1	25.9	3.9	15.1	0.0	0.0	0.0	0.0	3.9	15.1
Sample 2	32.7	3.7	11.2	0.0	0.0	2.0	6.0	4.2	12.7
Sample 3	46.1	3.5	7.5	0.0	0.0	1.2	2.6	3.7	7.9
Sample 4	76.4	3.2	4.1	0.0	0.0	1.7	2.3	3.6	4.7

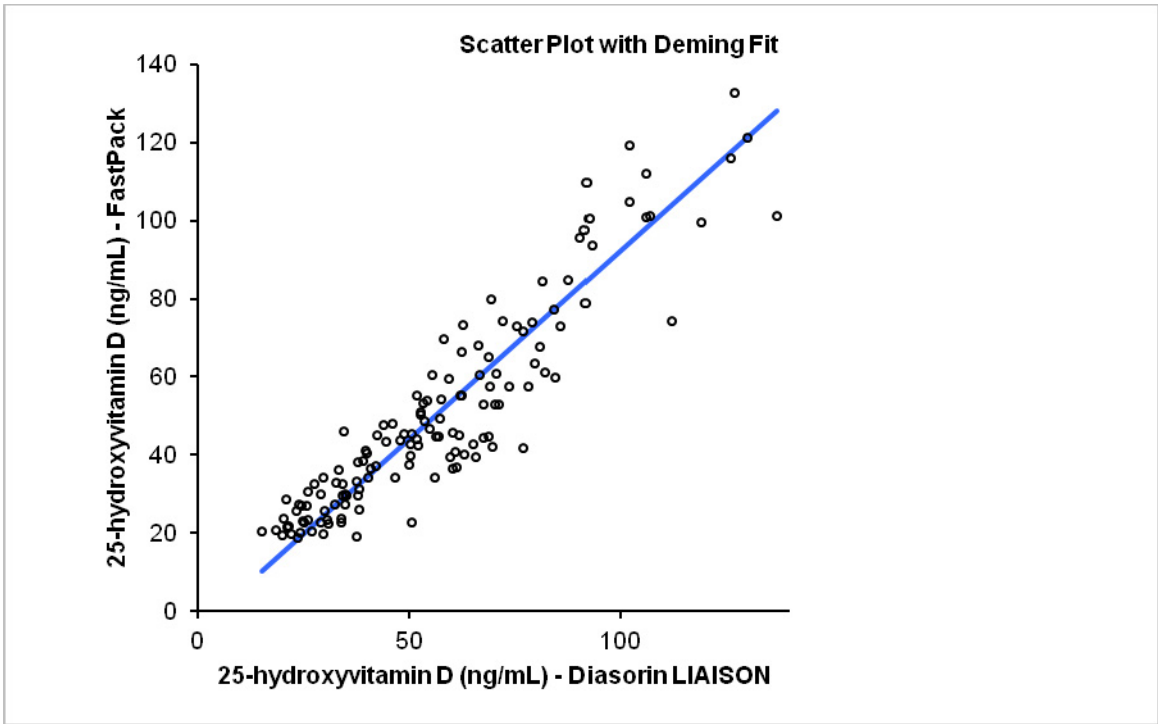
Range of linearity

For Vitamin D as tested by the FastPack® Vitamin D Immunoassay, the method has been demonstrated to be linear from the LOQ (12.9 ng/mL) to 150 ng/mL within 5 ng/mL in the interval.

Method Comparison

Clinical serum samples (n=137) were used to compare the values obtained using the FastPack® Vitamin D Immunoassay method and the values obtained using the DiaSorin LIAISON® Vitamin D TOTAL Assay method. The values were evaluated for agreement using Deming regression analysis, with the associated correlation coefficient.

n	Range of Observation (ng/mL)	Intercept (ng/mL)	Slope	R
137	18.6 – 132.6	-4.6	0.97	0.92



Interfering Substances

The effect of interferences on quantification of vitamin D was investigated by preparation of serum samples with low and high vitamin D concentrations with known concentrations of bilirubin, biotin, cholesterol, protein, hemoglobin, and lipids. The value obtained for the sample with each interfering substance was compared to the value obtained for the sample without the interfering substance and the percentage bias in ng/mL determined. These compounds did not show interference at the levels indicated.

	Interfering substance					
	Bilirubin (40 mg/dL)	Biotin (1 µg/mL)	Cholesterol (500 mg/dL)	Protein (10.7 g/dL)	Hemoglobin (500 mg/dL)	Lipids (250 mg/dL)
Non-spiked aliquot	59.1 ng/mL	36.0 ng/mL	38.5 ng/mL	28.2 ng/mL	49.0 ng/mL	98.2 ng/mL
Spiked aliquot	53.1 ng/mL	34.9 ng/mL	41.3 ng/mL	31.1 ng/mL	45.7 ng/mL	88.5 ng/mL
% Bias	-10.2	-3.1	7.3	10.3	-6.7	-9.9

Cross-reactivity

Two serum samples containing low and high Vitamin D concentrations were tested without and with added concentrations of potential cross-reacting compounds including 1,25-dihydroxy Vitamin D2; 1,25-dihydroxy Vitamin D3; Vitamin D2; Vitamin D3; 25-hydroxy Vitamin D2; 25-hydroxy Vitamin D3; 24,25-dihydroxy Vitamin D2; 24,25-dihydroxy Vitamin D; 3-epi-25-hydroxy Vitamin D3, and Paricalcitol. Maximum cross-reactivity at the cross-reactant concentration indicated was determined for each compound.

Cross-reactant	Concentration tested (ng/mL)	% Cross-reactivity
Vitamin D2	500	2.0
Vitamin D3	500	1.9
1,25-(OH)2-Vitamin D2	100	4.0
1,25-(OH)2-Vitamin D3	100	9.8
3-epi-25(OH) Vitamin D3	400	7.8
25 (OH) Vitamin D2	100	93.0
25 (OH) Vitamin D3	25	106.0
Paricalcitol	200	-1.2
24, 25 (OH)2 Vitamin D2	40	-0.9
24, 25 (OH)2 Vitamin D3	20	117.4

Limit of Blank (LOB), Limit of Detection (LOD), and Limit of Quantitation (LOQ)
The limit of blank (LOB, the highest measurement likely to be observed for a blank sample), limit of detection (LOD, the lowest amount of analyte in a sample that can be detected with type I and II error rates set to 5%), and limit of quantitation (LOQ, the lowest amount of analyte in a sample that can be reliably detected and at which the total error meets the pre-specified requirement for accuracy) were determined according to CLSI EP17-A for the FastPack® Vitamin D Immunoassay. In this study, the limit of blank was determined from 160 replicate determinations of a delipidated Vitamin D-free human serum on each of six different FastPack® instruments using three reagent lots. Raw RLUs from the assays were converted to apparent ng/mL based on the calibration curve for each assay. The LOB was determined as the upper 95th percentile of the distribution. This value was 2.3 ng/mL Vitamin D.

The LOD was estimated from 60 replicate determinations of four low samples. Per the CLSI EP17-A guideline, LOD was determined by the following equation:

$LOD = LOB + (c_ß * SD_S)$,

Where $c_ß = 1.645/(1-(1/(4 *f))$, where f is the degrees of freedom, and SD_S is the pooled standard deviation of the observations. In this study, the LOD was found to be 6.2 ng/mL Vitamin D.

For the LOQ analyses, % CV was plotted versus Vitamin D concentration and the concentration at which a 20% CV occurs based on a fit of the points was identified. LOQ was set to 12.9 ng/mL Vitamin D.

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