EXPERT REVIEWS

First 25-hydroxyvitamin D assay for general chemistry analyzers

Expert Rev. Mol. Diagn. Early online, 1–11 (2014)

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25-Hydroxyvitamin D [25(OH)D], the predominant circulating form of vitamin D, is an accurate indicator of the general vitamin D status of an individual. Because vitamin D deficiencies have been linked to several pathologies (including osteoporosis and rickets), accurate monitoring of 25(OH)D levels is becoming increasingly important in clinical settings. Current 25(OH)D assays are either chromatographic or immunoassay-based assays. These assays include HPLC, liquid chromatography-tandem mass spectrometry (LC-MS/MS), enzyme-immunosorbent, immunochemiluminescence, immunofluorescence and radioimmunoassay. All these assays use heterogeneous formats that require phase separation and special instrumentations. In this article, we present an overview of these assays and introduce the first homogeneous assay of 25(OH)D for use on general chemistry analyzers. A special emphasis is put on the unique challenges posed by the 25(OH)D analyte. These challenges include a low detection limit, the dissociation of the analyte from its serum transporter and the inactivation of various binding proteins without phase separation steps.

KEYWORDS: 25-hydroxyvitamin D • CLIA • Cloned enzyme-donor immunoassay • colorimetric detection • EIA • FIA • general chemistry analyzer • RIA • vitamin D binding protein

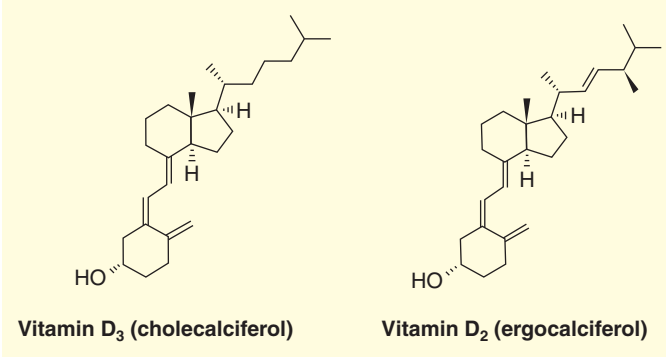
Vitamin D

Vitamin D is a group of anti-rachitic fat-soluble 9,10-secosteroids. The most important compounds of this group are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) (FIGURE 1). In this study, we use the term 'vitamin D' to exclusively refer to vitamin D_2 or vitamin D_3 compounds. Vitamin D_3 is synthesized in the skin when 7-dehydrocholesterol is exposed to ultraviolet B radiation from sunlight [1,2]. The exact amount of vitamin D_3 that is made by the skin is difficult to estimate or predict. It depends on several factors, such as the extent of the exposed skin surface, the length of exposure, the overall health of the skin, the skin tone, the seasonal variations in the intensity of ultraviolet B radiation and the number of ultraviolet B radiation-absorbing pigments in the skill cells. Sunscreens have been shown to dramatically interfere with the production of vitamin D_3 by the skin [1,3,4]. A recent research study showed that application of the SPF8 sunscreen, according to the manufacturers' recommended doses, completely blocked the production of vitamin D by the skin. Skin-synthesized vitamin D_3 can be complemented with vitamin D_3 from dietary sources such as fish (mainly salmon, sardines and tuna), liver oils and egg yolks. Vitamin D_2, a derivative of ergosterol, is produced by some phytoplankton, fungi (including yeast and certain mushrooms) and by a few invertebrate animals [5,6]. Land plants and vertebrates are incapable of producing vitamin D_2 because they lack the ergosterol precursor. Unlike vitamin D_3, which is available as dietary supplement, vitamin D_2 is a therapeutic drug in the USA, and can be only obtained through doctor's prescription.

Vitamin D metabolism & physiological roles

Vitamin D, synthesized in the skin and/or obtained from dietary supplements [7,8], circulates in the bloodstream bound to vitamin D binding protein (DBP) [9,10]. DBP is a glycosylated α-globulin of approximately 58 kDa, product of expression of the *GC* gene located on human chromosome 4 [11]. DBP-bound vitamin D is transported to the liver where it is converted by the enzyme cholecalciferol

 10.1586/14737159.2015.988144 ISSN 1473-7159



Vitamin D_3 (cholecalciferol) Vitamin D_2 (ergocalciferol)

Figure 1. The structures of (left) vitamin D_3 and (right) vitamin D_2.

25-hydroxylase into 25-hydroxyvitamin D (25[OH]D) (Figure 2). 25(OH)D has a half-life of several weeks [12]. Physicians measure 25(OH)D to evaluate the vitamin D status of a patient.

25(OH)D undergoes an additional hydroxylation catalyzed by the enzyme 25-hydroxyvitamin D-1-α-hydroxylase to produce 1,25-dihydroxyvitamin D (Figure 2). This metabolite serves as the active form of vitamin D, fulfilling many of its physiological functions. The half-life of 1,25-dihydroxyvitamin D is only a few hours, which makes it an inaccurate marker for the general vitamin D status of an individual [12].

The hydroxylation of 25(OH)D occurs mainly in the kidneys where the produced 1,25-dihydroxyvitamin D is involved in bone health and calcium homeostasis. It is established, indeed, that 1,25-dihydroxyvitamin D enhances the intestinal absorption of calcium and phosphates (which are important for bone calcification) as well as the intestinal absorption of other oligoelements such as iron, magnesium and zinc [13]. 1,25-Dihydroxyvitamin D produced by the kidneys is also important for muscle health, immunomodulation, neurodevelopment, cardiovascular health and blood pressure regulation [14].

The conversion of 25(OH)D into active form can also occurs in white blood cells (monocytes and macrophages) where the production of 1,25-dihydroxyvitamin D is involved in immunomodulation, the prevention of autoimmune diseases and the control of invading pathogens [15]. It has been shown that 25(OH)D hydroxylation into 1,25-dihydroxyvitamin D can also occur in various organs and tissues (such as the prostate gland, breast, colon, lung and keratinocyte-containing tissues) where it serves as a regulator of cell growth and cell differentiation, hence its putative anti-oncogenic effect [16].

The need for a vitamin D test

A growing body of research has been highlighting (since the 1990s) the importance of having sufficient levels of 25(OH)D. Depending on serum calcium levels, insufficient levels of the circulating form of vitamin D have been associated with increased levels of the parathyroid hormone [17]. Parathyroid hormone increases bone resorption leading to bone loss. A positive association exists between serum 1,25-dihydroxycholecalciferol levels and bone mineral density [17]. A recent study of 177 patients with various oncologic diagnoses showed the prevalence of vitamin D deficiencies and suggested that patients who are at high risk for vitamin D deficiency or poor bone health to be regularly screened for various cancers [18]. The impact of vitamin D in breast cancer [19], rheumatoid arthritis [20], prostate hyperplasia [21], hypertension risk [22], mortality in stroke patients [23], postpartum depression [24], obesity [25] and insulin resistance [26] has been also suggested and discussed.

Serum levels of 25(OH)D have been shown to have seasonal variation of up to 40% (highest levels are found measured during the summer season, lowest levels are measured during the spring season) [27,28]. Despite these variations, 25(OH)D deficiency is very common. The study of a US population aged 1 to 70+ years old showed that over 30% of females and over 25% of males were vitamin D deficient [29]. A 2005–2006 National Health and Nutrition Examination Survey analyzed the 25(OH)D levels of nearly 4500 participants. The survey found that 41.6% of participants were deficient (having 20 ng/ml or less of serum 25(OH)D). African Americans had the highest deficiency rate (82.1%) followed by Hispanics (69.2%) [30–32].

The association between several pathologies and vitamin D deficiencies highlights the importance of developing assays that measure 25(OH)D. As the hydroxylation of either vitamin D_2 or vitamin D_3 (by the same enzymes) leads to equally active vitamin D metabolites, measuring total 25(OH)D (sum of plasma or serum circulating 25-hydroxyvitamin D_2 [25(OH)D_2] and 25-hydroxyvitamin D_3 [25(OH)D_3]) is considered an essential indicator of the vitamin D status of an individual [33].

The analytical challenge of measuring total 25(OH)D

To accurately measure the total 25(OH)D level of an individual, the assay must first recognize 25(OH)D_2 and 25(OH)D_3 equally. This is a particularly challenging task. Indeed, 25(OH)D_2 and 25(OH)D_3 have subtle structural differences, have different binding affinities to their natural serum transporter (DBP) and have different half-lives [34,35]. In addition, the assay must not significantly cross-react with a multitude of vitamin D metabolites (including vitamin D_2 and vitamin D_3 and 3-epivitamin D [36]) despite these metabolites being structurally very similar to 25(OH)D. As 25(OH)D is a highly hydrophobic molecule, it exclusively circulates bound to DBP (free circulating 25(OH)D has never been found). An assay that measures 25(OH)D needs, therefore, to dissociate this analyte from its transporter before quantification. This represents another technical challenge because not only the affinity of DBP to 25(OH)D is relatively high ($K_a = 5.10^{+8}$) but also serum levels of DBP are in large excess compared with 25(OH)D. It is estimated that less than 5% of the available DBP binding sites are occupied with vitamin D. Moreover, it has been shown that serum DBP levels can vary drastically among patients (from as low as 90 μg/ml to as high as 1100 μg/ml in an apparently normal population) [37]. It is also known that several highly abundant serum albumins (i.e., HSA) may non-specifically bind vitamin D metabolites once released from DBP. The abundance of unoccupied DBP

doi: 10.1586/14737159.2015.988144

and the non-specific interaction with other serum albumins complicates the techniques of release of 25(OH)D for analytical purposes. For several years, detecting 25(OH)D required total and irreversible denaturation of serum proteins with organic solvents (mostly acetonitrile) [38]. Unfortunately, the use of these organic solvents is mostly incompatible with the design of a fully automated, high-throughput 25(OH)D assay.

Overview of the current market for 25(OH)D testing

25(OH)D testing has increased exponentially in the past 10 years. The worldwide vitamin D market size was only 12 millions tests in 2007. In 2011, it reached 80 million tests. It is estimated that vitamin D testing will grow at the rate of 32% over the next 4 years (2014–2018). This increase is attributed mainly to the substantial number of research articles highlighting the physiological importance of vitamin D and to the increase in size of the chronically ill, aging populations. To-date, most vitamin D testing is performed in the USA (156 tests/1000 inhabitant), Europe (150 tests/1000 inhabitant in Belgium for example) and Australia (148 tests/1000 inhabitant). Current vitamin D testing in Asia and Africa is lagging behind (less than 1 test/1000 inhabitant) but is expected to grow exponentially in the upcoming few years.

25(OH)D has been routinely measured by HPLC, RadioImmunoAssay (RIA) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods [39–41]. In particular, the LC–MS/MS method [42,43] (chosen as the reference method by the US Centers for Disease Control) is the only method capable of reporting the individual concentrations of $25(OH)D_2$, $25(OH)D_3$ and of their corresponding C3 epimers [44,45]. However, despite their accuracy and sensitivity, the methods listed above struggled to meet the growing demand for 25(OH)D testing. HPLC, RIA and LC–MS/MS are indeed labor intensive, time-consuming, require highly trained technicians and lack full-automation. As a result, testing throughputs are low and turnaround times for results are long (over 24 h).

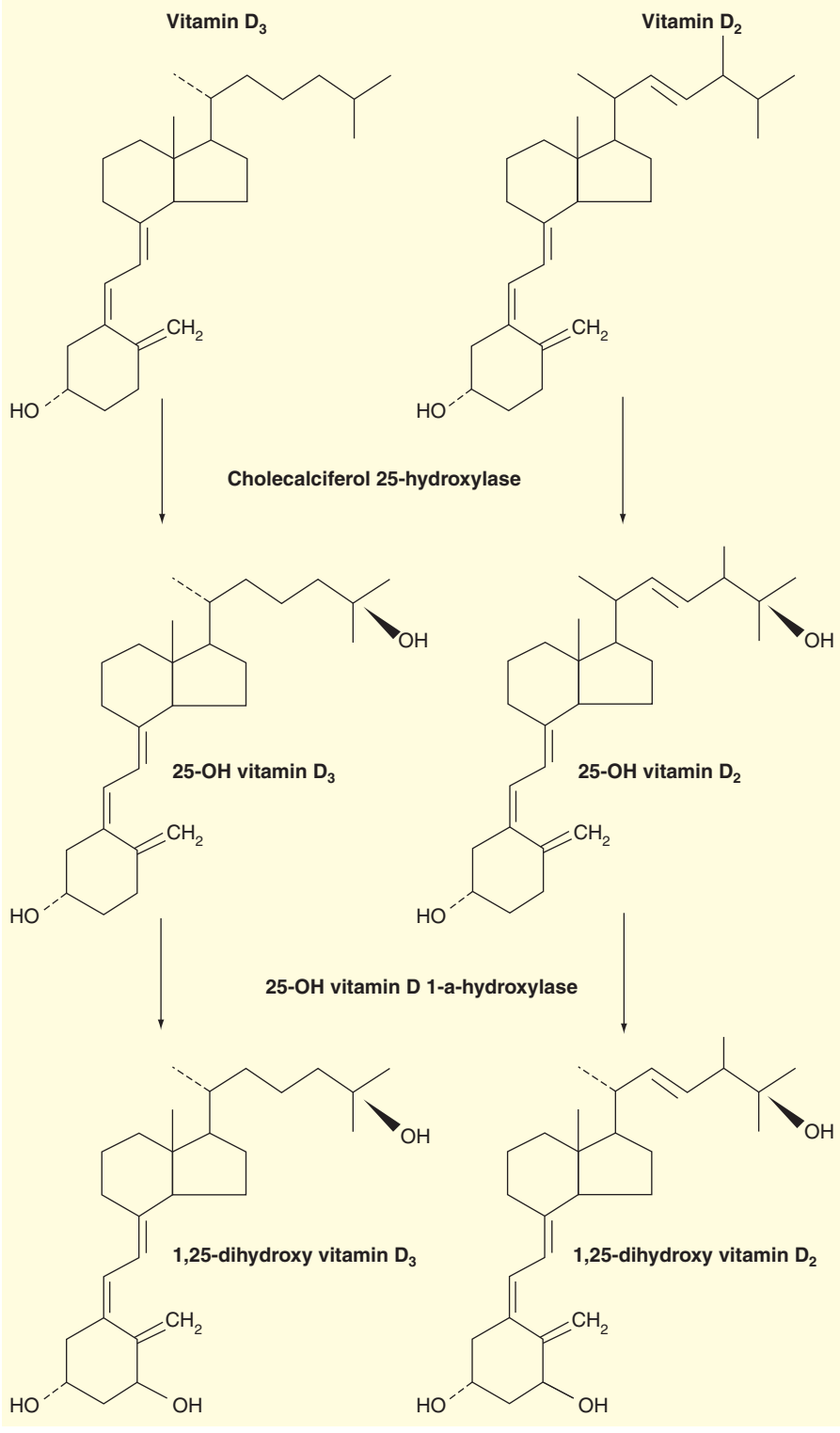


Figure 2. Conversion of vitamin D to $25(OH)D_2$ and $25(OH)D_3$ and to 1,25-dihydroxyvitamin D_2 and 1,25-dihydroxyvitamin D_3.

Since the early 2000s, the US FDA has approved several fully automated immunoassays methods for 25(OH)D [46]. The vast majority of these immunoassays use a common core technology based on magnetic beads and chemiluminescence detection. The quality of these automated assays varies between manufacturers. Although, these assays are fully automated and

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Table 1. 25-Hydroxyvitamin D assays approved by the US FDA.

Device name	510k	Manufacturer	Technology	Range (ng/ml)	Sample type	Time to 1st result	Platform/analyzer
Diazyme 25(OH)D assay	K133410	Diazyme Laboratories	Homogenous enzymatic immunoassay with colorimetric detection	7.6–147.8	Serum or plasma	21 min	Roche Modular P and similar
25(OH)D Elisa	K123660	Euroimmun US	ELISA	2.4–144	Serum or plasma	3.5 h	ELISA plate reader
25(OH)D Total ELISA Test	K123364	Diasource Immunoassays S.A	ELISA	7.7–122.9	Serum only	4.0 h	ELISA plate reader
Fastpack vitamin D immunoassay	K123983	Qualigen, Inc.	Direct competitive chemiluminescence immunoassay	12.9–150	Serum or plasma	10 min	FastPack® Analyzer
Vitros 25(OH)D total Assay	K121608	Ortho-Clinical Diagnostics, Inc.	Solid phase antibody capture immunoassay	12.8–126	Serum only	24 min	VITROS® ECi/ECi, 3600, 5600
ST AIA-Pack 25(OH)D	K123131	Tosoh Bioscience, Inc.	Fluorescence immunoassay	4–120	Serum or plasma	40–50 min	AIA-600II, AIA-900 AIA-1800, AIA-2000
Diazyme 25-hydroxy vitamin D EIA kit	K122420	Diazyme Laboratories	ELISA	8.3–143.6	Serum or plasma	2.0 h	ELISA plate reader
Elecsys vitamin D assay	K113546	Roche Diagnostics	Electrochemiluminescence quantitative protein binding assay	5–60	Serum or plasma	27 min	Cobas e411 Analyzer
Liaison 25 OH vitamin D Total Assay	K112725	Diasorin, Inc.	Chemiluminescent immunoassay	4–150	Serum only	35 min	Liaison Analyzer
Architect 25-OH vitamin D	K110619	Biokit S.A.	Chemiluminescent microparticle immunoassay	13.0–96.0	Serum or plasma	36 min	Abbott Architect I
Advia Centaur vitamin D Total Assay	K110586	Siemens Healthcare Diagnostics Inc.	Chemiluminescent competitive immunoassay	4.2–150	Serum or plasma	18 min	The ADVIA Centaur XP instrument
IDS-Isys 25-hydroxy vitamin D Assay	K091849	Immunodiagnostic Systems, Inc.	Quantitative chemiluminescent immunoassay	6–126	Serum only	38 min	IDS iSYS
ESA Biosciences Inc. vitamin D HPLC Test	K072536	Esa Biosciences Inc.	HPLC	7–200	Serum or plasma	>1 h	ESA HPLC system
Octeia 25-hydroxy vitamin D	K021163	Immunodiagnostic Systems Ltd.	ELISA	2.4–144	Serum or plasma	3.5 h	ELISA plate reader
25-Hydroxyvitamin D 125i Ria Kit	K983617	Diasorin, Inc.	RIA	N/A	Serum or plasma	2.0 h	RIA system

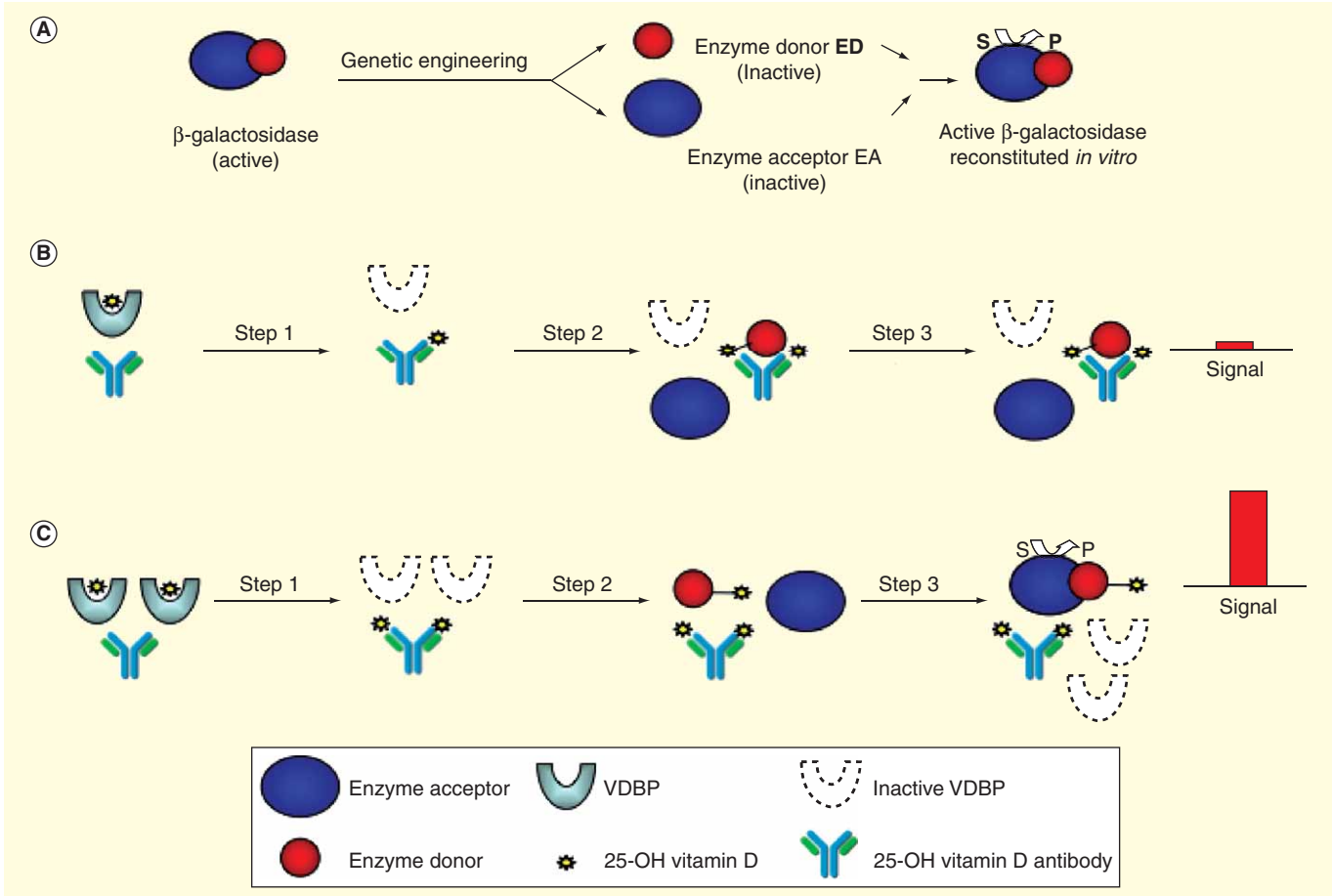

Figure 3. Principle of the Diazyme 25(OH)D assay. (A) The β-galactosidase α-complementation system. By means of genetic engineering, β-galactosidase is split in two inactive parts (the enzyme donor and the enzyme acceptor). When combined with the enzyme donor (or any enzyme donor conjugate), the enzyme acceptor regains enzymatic activity and is capable of generating a measurable signal. **(B)** Signal development in a low vitamin D specimen. Vitamin D is first dissociated from its transporter vitamin D binding protein during Step 1. The 25 (OH)D antibody is not fully saturated and is able to complex the enzyme donor conjugate, hindering its complementation of the enzyme acceptor in Steps 2 and 3. Only a low background signal is developed and **(C)** signal development in a high vitamin D specimen. Vitamin D is first dissociated from its transporter vitamin D binding protein during Step 1. The 25(OH)D antibody is now fully saturated and is unable to complex the enzyme donor conjugate. The free enzyme donor conjugate is capable of complementing the enzyme acceptor and generating a strong colorimetric signal during Steps 2 and 3.

use systems easy to operate, they still involve multiple phase-separation steps (washing steps) and require special instruments or manufacturer-specific instruments to run exclusively the analyte 25(OH)D. In addition, some of these immunoassays do not measure 25(OH)D_2 and 25(OH)D_3 equally, have poor correlation with the reference method (LC–MS/MS), and poor traceability to the National Institute of Standards and Technology (NIST) standard reference material SRM972 [47]. Table 1 lists the major assays for 25(OH)D currently approved by the FDA.

Description of the diazyme assay for 25(OH)D

The assay is based on the principle of α-complementation [48] of the enzyme β-galactosidase and the competition between an enzyme donor–25(OH)D conjugate, an anti-vitamin D antibody and the 25(OH)D content of a serum sample. The assay's principle is depicted in Figure 3.

The assay kit consists of five serum calibrators (containing assigned amounts of 25(OH)D), a sample diluent and three reagents R1, R2 and R3. These reagents are composed of β-galactosidase enzyme acceptor, a β-galactosidase enzyme donor–vitamin D conjugate, an anti-vitamin D antibody and a β-galactosidase substrate (an ortho-nitrophenyl-β-galactoside analog). All kit components are liquid stable and ready to use.

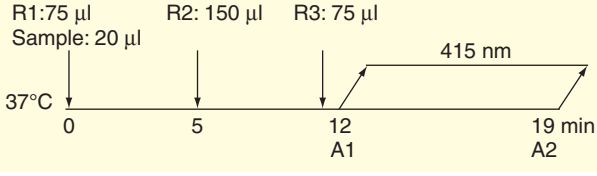

Figure 4. The programmable sequence for the Diazyme 25 (OH)D assay on general chemistry analyzers.

doi: 10.1586/14737159.2015.988144

Table 2. Method comparison between the diazyme assay and the diasorin liaison assay.

Deming regression analysis	95% CI
Slope	1.005 (0.969–1.041)
Intercept	-0.21 (-2.15 to 1.73)
Correlation coefficient	0.984 (0.976–0.989)
Range	9.5–140.9

Specimens are first diluted, on-board, with the provided sample diluent. In a suitable cuvette, 20 µl of the diluted specimen are mixed with 75 µl of reagent R1 and incubated for 5 min at 37°C. During this step, vitamin D metabolites are dissociated from their transporter (vitamin D binding protein). Subsequently, 150 µl of reagent R2 are added to the mixture and immuno-competition is allowed to proceed for 5 min. Finally, 75 µl of reagent R3 are added to allow for β-galactosidase signal development. A nitro-phenyl-β-galactoside derivative is used as the enzyme substrate. The reaction's product has a maximum absorbance at 415 nm. This absorbance is proportional to the concentration of 25(OH)D in a particular specimen. A schematic representation of the assay procedure is shown in Figure 4.

Performance of the assay

The performance of the Diazyme 25(OH)D assay has been fully validated for the use on Roche's Modular P general chemistry analyzer. The assay parameters were also successfully developed for applications of the assay to other models of chemistry analyzers such as Integra 400, Pentra 400, Ace Alera, Hitachi 911 and Mindray BS-480. The results reported are expressed in ng/ml. The dynamic range of the assay is 7.6–147.8 ng/ml (samples with values greater than 147.8 ng/ml are reported as >147.8 ng/ml, samples with values less than 7.6 ng/ml are reported as <7.6 ng/ml). The assay's throughput is approximately 100 tests/h, with the first results being reported in less than 30 min. Once the application parameters are programmed into a general chemistry analyzer channel, the assay can be run by any technician with the most basic clinical chemistry skills. No special training is required. Waste disposal follows the standard procedures in place in any typical clinical chemistry laboratory. Analytical performance, comparison studies and reference studies are shown below.

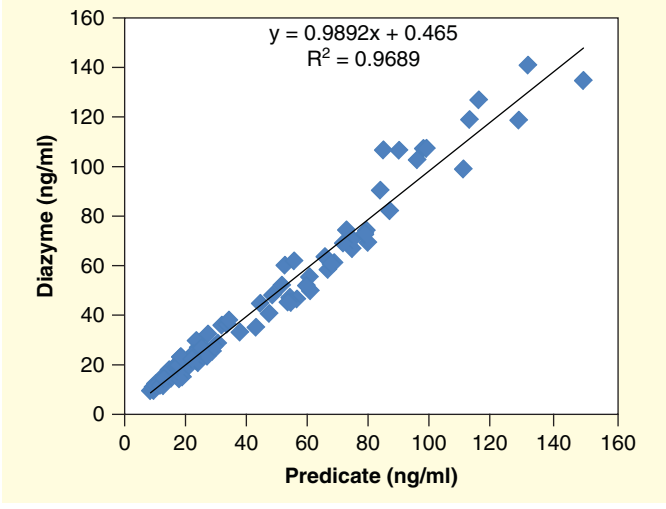

Figure 5. Linear regression plot of the Diazyme versus Predicate 25(OH)D method comparison.

Sensitivity

Limits of detections were determined according to the Clinical and Laboratory Standards Institute (CLSI) EP17-A guideline. The limit of blank, the limit of detection and the limit of quantification were 2.0, 3.5 and 7.6 ng/ml, respectively,.

Accuracy

The performance of this assay was compared with the performance of The DiaSorin Liaison Assay (DiaSorin Inc.). The results of 98 serum samples are shown in Table 2. Linear regression plot of method comparison is shown in Figure 5.

Matrix comparison

To evaluate the effect of anticoagulants, the Diazyme 25(OH)D assay was used to measure the 25(OH)D concentrations of matched sets of serum, potassium ethylenediaminetetraacetic acid (K3-EDTA) plasma and Li-heparin plasma. All three matrices can be used for the assay. Indeed, linear regression of the 'K3-EDTA plasma versus serum' data yielded the following results: y = 0.9948x – 0.7057 and R2 = 0.9866. Linear regression of the 'Li-heparin plasma versus serum' data yielded the following results: y = 0.9657x – 0.6596 and R2 = 0.9736. Figure 6 shows the linear plots of these matrix comparison studies.

Precision

Precision was evaluated according to the CLSI EP5-A guideline. Controls and samples were measured daily over the span of 20 days, using three lots of reagents. Forty independent runs were performed on each specimen. Each run produced two measurements. Eighty data points were obtained per specimen. Results are shown in Table 3.

Linearity

Eleven levels of linearity were prepared by diluting a high serum sample with vitamin D-depleted serum. Linearity levels were prepared according to the CLSI EP6-A guideline. The assay was found to be linear between 7.6 and 147.8 ng/ml. Figure 7 shows the linearity plot of the Diazyme 25(OH)D assay.

Interference

Interference studies were conducted according to the CLSI EP7-A2 guideline. The acceptance criterion was set at 10% or less deviation between the spiked sample and the control. The assay's results were not significantly affected by following compounds listed in Table 4.

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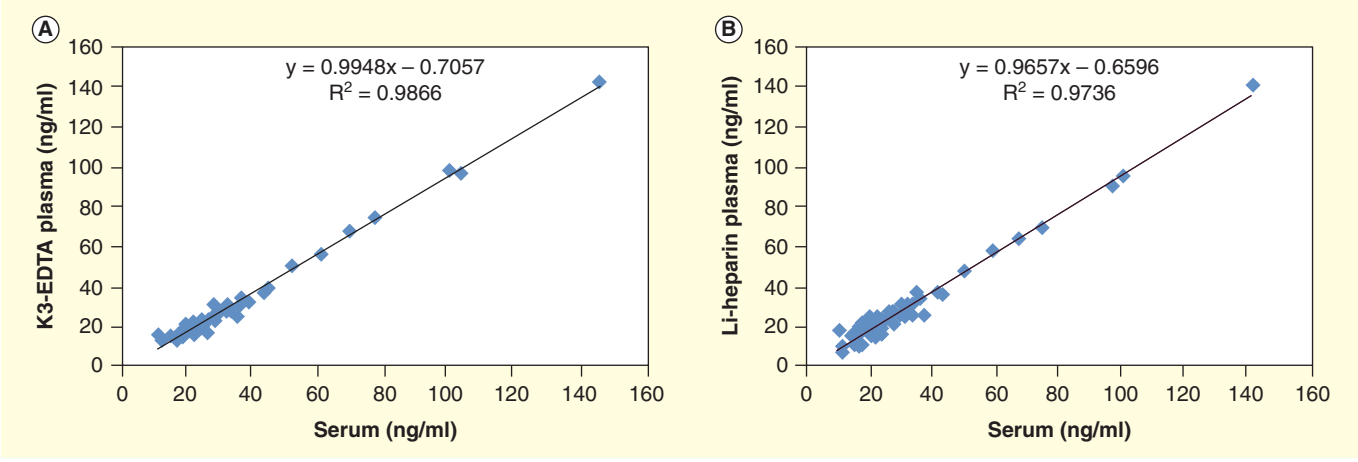

Figure 6. Matrix comparison linear regression plots. (A) K3-EDTA plasma versus serum and **(B)** Li-Heparin plasma versus serum. K3-EDTA: Potassium ethylenediaminetetraacetic acid.

Cross-reactivity

Cross-reactivity of the Diazyme 25(OH)D assay was determined by adding vitamin D metabolites to serum pool samples. Based on the results in the table, the assay showed no preference in detecting vitamin D_3 versus vitamin D_2, and the assay recovers both 25(OH)D_3 and 25(OH)D_2 similarly. Cross-reactivity with various vitamin D metabolites is summarized in Table 5. No significant cross-reactivity (4.1%) was found for paricalcitol (Zemplar®) up to 25 ng/ml.

Our assay cross-reacts to 92% with 25(OH)D_2 and 100% with 25(OH)D_3. This small difference in cross-reaction is likely artifactual because of uncertainties in the quantification of spiked 25(OH)D_2 and spiked 25(OH)D_3, small differences in the solubility, small differences in the stability or small differences in the purity of these two vitamins. Our assay cross-reacts with the 3-epi-25-hydroxyvitamin D epimers to 51–62%, making it unsuitable for measuring neonatal samples.

Because anomalous behavior of exogenously added material (compared to endogenous material) has been observed by several clinical diagnostics manufacturers, we cannot completely exclude that the reported endogenous material interference and cross-reactivity might be slightly different from those obtained exclusively with endogenous material.

Table 3. Within-run, between-run and total assay precision.

25(OH)D (ng/ml)			Within-run		Between-run		Total	
Specimen	*n*	*Mean*	*SD*	*%CV*	*SD*	*%CV*	*SD*	*%CV*
Control #1	80	23.1	1.47	6.4	1.04	4.5	1.68	7.3
Control #2	80	45.7	2.06	4.5	1.67	3.7	2.12	4.6
Sample #1	80	22.6	1.19	5.3	1.11	4.9	1.45	6.4
Sample #2	80	31.7	1.42	4.5	1.59	5.0	1.81	5.7
Sample #3	80	40.6	1.42	3.5	1.59	3.9	1.66	4.1
Sample #4	80	48.6	2.32	4.8	1.71	3.5	2.41	4.9
Sample #5	80	55.8	2.14	3.8	1.73	3.1	2.34	4.2
Sample #6	80	65.4	2.03	3.1	1.79	2.7	2.42	3.7
Sample #7	80	69.7	2.02	2.9	1.99	2.9	2.55	3.7
Sample #8	80	92.8	2.52	2.7	2.02	2.2	3.40	3.7
Sample #9	80	134.6	2.97	2.2	2.69	2.0	3.87	2.9
Low sample #1	80	9.4	1.22	13.0	0.98	10.4	1.31	14.0
Low sample #2	80	11.2	1.58	14.2	0.88	7.9	1.55	13.9

CV: Coefficient of variation; SD: Standard deviation.

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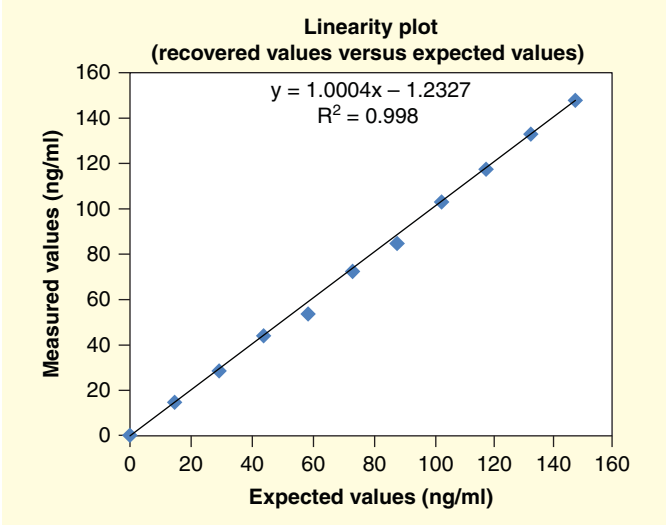

Figure 7. Linearity plot: The plot shows the recovered values (obtained in an 11-level dilution experiment) versus the expected values.

Reference range

Reference range of the Diazyme 25(OH)D assay was determined by measuring the 25(OH)D serum concentrations of the US population of 157 apparently healthy adults, 21–80 years old, during the months of October and November (fall season). Individuals were from three different geographical locations: 47 from Pennsylvania (Northern US), 56 from Tennessee (Central US) and 54 from Texas (Southern US). All 157 individuals did not have kidney disease, GI disease, liver disease, calcium level-related disease, thyroid disease, parathyroid disease, seizures, chronic disease or bariatric surgery. The 2.5–97.5th percentile range was 15.0–45.9 ng/ml. The median concentration was 25.6 ng/ml.

Table 4. Assay tolerance to various interference substances.

Substance	Concentration (mg/dl)
Conjugated bilirubin	40
Free bilirubin	40
Hemoglobin	100
Ascorbic acid	176
Triglycerides	750
Uric acid	20
Biotin	2
Human serum albumin	9
N-acetyl cysteine amide	1663
Ampicillin	1000
Cyclosporine C	105
Cefoxitin	660
Acetylsalicylic acid	1000
Rifampicin	64
Acetaminophen	200
Ibuprofen	500
Theophylline	100

Regulatory affairs

The Diazyme assay for 25(OH)D was cleared by the FDA on 14 March 2014 (510k number k133410). The assay is also CE marked. The product is now being marketed in the USA, Europe and Asia.

Conclusions

Because vitamin D deficiencies have been linked to many diseases (including osteoporosis, rickets, cardiovascular diseases and cancers), accurate monitoring of total 25(OH)D levels is important in clinical settings. Currently available 25(OH)D assay methods include LC–MS/MS, radioimmunoassay, ELISA and chemiluminescent immunoassays using special instruments. None of these assays runs on clinical chemistry analyzers that are commonly available in hospitals and reference laboratories. In 2014, Diazyme introduced a homogenous 25(OH)D assay using an enzyme–antibody combination technique. It is the first fully automated colorimetric assay for 25(OH)D that runs on general clinical chemistry analyzers (e.g., Roche Modular P). The Diazyme assay is expected to allow clinical laboratories of all sizes to run 25(OH)D tests in-house. The Diazyme assay was cleared by the FDA on 14 March 2014. It was also CE marked at the end of 2013. The product is now being marketed worldwide.

Expert commentary

With the Diazyme 25(OH)D assay, vitamin D testing can now be performed on general chemistry analyzers using routine colorimetric detection. This technological breakthrough was made possible, thanks to several developments.

First, several high-affinity and high-specificity 25(OH)D antibodies were made available over the past few years. This allowed Diazyme to perform the competition step (between analyte, antibody and enzyme–donor conjugate) over a relatively short period of time (1–5 min, compatible with the timing needed for chemistry analyzers). This short competition largely contrasts with early immunoassays for 25(OH)D where lengthy competition steps lasted between 2 and 18 h (overnight incubation).

Second, as there is no clinical significance in measuring 25(OH)D_2 and 25(OH)D_3 separately, the development of antibodies that recognize 25(OH)D_2 and 25(OH)D_3 equally was important in reporting the true total 25(OH)D (unbiased sum of 25(OH)D_2 + 25(OH)D_3). In the early 2000s, original 25(OH)D assays suffered from the lack of complete cross-reaction with 25(OH)D_2 (which could be as low as 75%).

Third, a significant improvement to the cloned-enzyme donor technology has contributed to Diazyme's new method for detecting vitamin D. β-Galactosidase enzyme donor and enzyme acceptor fragments were specially engineered to detect

doi: 10.1586/14737159.2015.988144

25(OH)D. The enzyme donor was modified to accommodate a single, site-specific, conjugation to vitamin D. The conjugation site was designed to minimize the interference with the activity of the enzyme and promote the recognition of the vitamin D epitope by a specific 25(OH)D antibody. The enzyme acceptor was also engineered to sustain a high turnover rate once enzyme donor and enzyme acceptors reconstitute the active β-galactosidase. In the Diazyme assay, enzyme donor and enzyme acceptor are part of liquid-stable, ready-to-use reagents. This contrasts with earlier cloned enzyme donor immunoassays in which all reagents were lyophilized and needed reconstitution with special buffers.

Dissociating vitamin D from its abundant serum transporter (DBP) has been one of the major challenges for automated assays for 25(OH)D. Diazyme uses its organic solvent-free proprietary diluent to irreversibly dissociate vitamin D. This dissociation happens in a single aqueous and homogenous step. No solid phases are involved (i.e., magnetic beads, ELISA solid surfaces and the like) and no wash steps are needed which made the assay adaptable to general chemistry analyzers.

Finally, achieving the detection limit required for 25(OH)D testing was made possible, thanks to the use of Diazyme's proprietary β-galactosidase substrates that combine high extinction coefficients with long-term liquid-stability in manufactured reagents. Commercially available substrates could not be used for the assay because they are either liquid stable but have a low extinction coefficient (i.e., ortho-nitrophenyl-β-galactoside [ONPG]) or have a high extinction coefficient but are liquid unstable (i.e., chlorophenol red-β-D-galactopyranoside [CPRG]).

Early assays for 25(OH)D suffered from several performance issues, chief among them was poor method-to-method accuracy. This was due to several factors that include the use of different molecular competitors (vitamin D binding protein versus antibodies with various molecular specificities and cross-reactivities), the permanent removal of serum proteins (HPLC, RIA, LC–MS/MS) versus the non-permanent removal of serum proteins (ELISA, chemiluminescence immunoassays, chemiluminescence competitive protein binding assays and colorimetric immunoassays). The introduction of NIST-972 reference material in 2008 (four serum levels with LC–MS/MS certified values for 25(OH)D_2, 25(OH)D_3 and 3-epi-25-hydroxyvitamin D_3) helped improve the accuracy of the current 25(OH)D assays. However, significant between-methods accuracy issues still remain. It is not unusual to find, in 2014, a leading 25(OH)D assay displaying large biases (ranging between -21 and +56%) in the 95% agreement interval with the LC–MS/MS reference values [49,50]. These accuracy issues are very likely due to assay-dependent and assay-independent interference and/or cross-reacting substances that are occasionally present in the patient's serum. These substances are yet to be discovered and solutions counteracting their effects have yet to be invented.

Five-year view

25(OH)D testing has increased exponentially in the past decades. Vitamin D testing is expected to grow at the rate of 32% over the upcoming 4 years (2014–2018). A large body of research highlighting the physiological importance of 25(OH)D sustains this growth. This increasing demand for vitamin D testing faced, until now, several technological challenges. Chief among these challenges are full automation, high throughput, requirement for special and expensive equipment, as well as high cost per test for the reagents. Therefore, as of today, large hospital laboratories and reference laboratories are performing most of the 25(OH)D testing. Smaller laboratories have to send their specimens out to larger laboratories for vitamin D testing. The introduction of the Diazyme 25(OH)D assay for general clinical chemistry analyzers eliminates the need to acquire special equipment and allows laboratories of all sizes to run vitamin D testing using their existing chemistry analyzers (used for routine chemistry). This will not only improve the turnaround time for test results but will also significantly reduce the cost per test. The Diazyme assay will therefore quickly change the way clinical testing for 25(OH)D is performed. It will transform the 25(OH)D test into an affordable, routine clinical chemistry test contrasting with its current expensive and 'special chemistry' status. The fully automated, user friendly and cost–effective Diazyme 25(OH)D assay will help meet the growing demand for vitamin D testing in the USA as well as the rest of the world. Because of the physiological importance of vitamin D, a widely available 25(OH)D test should improve the general health of individuals through regular and targeted preventive care. It should also help reduce the cost of healthcare in the USA through a decrease in the size of the clinical population.

Table 5. Assay cross-reactivity with various vitamin D metabolites.

Compound	Concentration tested (ng/ml)	Cross-reactivity (%)
25(OH)D_3	44.0	100
25(OH)D_2	44.0	92.3
Vitamin D_3	44.0	1.0
Vitamin D_2	44.0	2.9
1,25-(OH)2 vitamin D_3	2.9	2.5
1,25-(OH)2 vitamin D_2	2.9	-1.5
24R,25-(OH)2 vitamin D_3	41.0	5.1
3-epi-25-hydroxyvitamin D_3	42.0	61.7
3-epi-25-hydroxyvitamin D_2	42.0	55.1

Financial & competing interests disclosure

All authors are employees of Diazyme. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

doi: 10.1586/14737159.2015.988144

Key issues

- 25-Hydroxyvitamin D [25(OH)D] is the accurate indicator of the general vitamin D status of an individual.
- 25(OH)D deficiencies are linked to many diseases.
- 25(OH)D testing has been growing exponentially in the past decade.
- Currently available assays for 25(OH)D are expensive, require special equipment, have low throughput and cannot be run in standard clinical chemistry labs.
- To meet the increasing demand for vitamin D testing, Diazyme developed the first fully automated colorimetric assay for 25(OH)D that runs on general chemistry analyzers.
- The Diazyme assay is poised to transform 25(OH)D into an affordable routine clinical chemistry test.
- Identifying and counteracting the effect of previously unknown 25(OH)D substances is essential to improve the accuracy and the agreement between various 25(OH)D assays.

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