

# ***VITAMIN D***

## ***beyond the bone***

***New Findings and New Test***

**Diazyme's 25-OH Vitamin D Assay for Clinical Chemistry Analyzers**



***INNOVATIONS IN CLINICAL DIAGNOSTICS***

## About Diazyme

Diazyme Laboratories is a Division of General Atomics located in Poway, California. Diazyme uses its proprietary enzyme and immunoassay technologies to develop diagnostic reagents which can be used on most automated chemistry analyzers in user-friendly formats. Diazyme is a cGMP and ISO 13485 certified medical device manufacturer. Diazyme's products include test kits for diagnosis of cardiovascular disease, liver disease, cancer markers, renal disease, diabetes and electrolytes.



### **MISSION STATEMENT**

Our mission is to improve the quality of healthcare by providing innovative products in clinical diagnostics.

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# 1. What is Vitamin D?

## 1.1 Vitamin D Molecules and Metabolism

Vitamin D is a steroid-like, fat soluble pro-hormone. Vitamin D has two main forms: D2 (ergocalciferol) and D3 (cholecalciferol). Vitamin D3 is synthesized in the skin by exposure to sunlight (ultraviolet radiation) and obtained from the diet primarily from fish, liver oils, and egg yolks. Vitamin D2 is obtained mainly from nutritional supplements. Vitamin D3 or D2 is metabolized in the liver to 25-hydroxy Vitamin D (25-(OH)D), which is then converted in the kidneys to 1,25-(OH)<sub>2</sub>D. Of the major circulating forms, 25-OH Vitamin D reflects the levels of Vitamin D in the body.

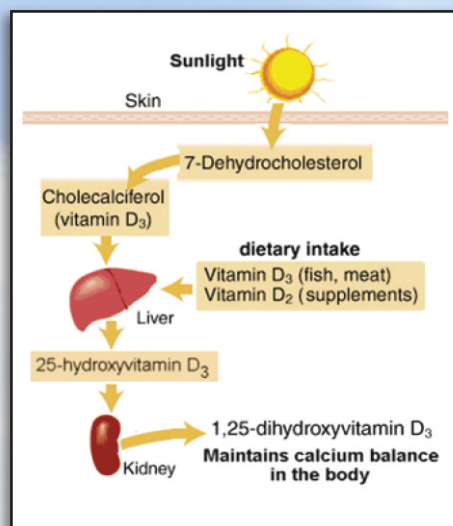


Figure 1

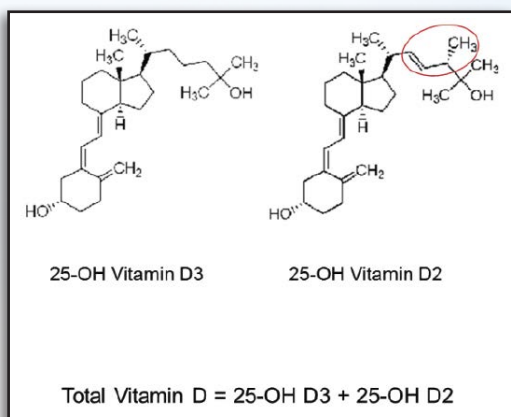


Figure 2

In the blood, Vitamin D exists as a protein bound form, specifically with a Vitamin D binding protein. Clinically, Vitamin D levels in the blood get reported as the total 25-OH Vitamin D, which is the sum of 25-(OH) D3 and 25-(OH) D2 (Figure 2).



## 2. Vitamin D Deficiency

### 2.1 Vitamin D Reference Range and Optimal Concentration

According to published literature, 25-(OH)D levels less than 10 ng/mL is considered evidence of severe Vitamin D deficiency, which could be associated with osteomalacia or rickets. 25-(OH)D serum level of 30 to 32 ng/mL is considered sufficient, whereas levels between 10 and 29 ng/mL are considered insufficient. At the present time, 25-(OH)D levels in the range of 30 to 60 ng/mL are considered optimal. Higher concentration levels of 100 ng/mL is often seen in individuals who receive intense sun exposure, such as individuals with outdoor occupations. According to the studies published in the New England Journal of Medicine (M. Holick, 2007)<sup>1</sup> and in the American Journal of Clinical Nutrition (J. Hathcock, 2007)<sup>2</sup>, serum level of 25-(OH)D below 20ng/mL is considered deficiency, and over 150 ng/mL is toxic (Tables 1 and 2).

Deficiency	< 10 ng/mL	(0 - 25 µmol/L)
Insufficiency	10 - 30 ng/mL	(25 - 75 µmol/L)
Sufficiency	30 - 100 ng/mL	(75 - 250 µmol/L)
Toxicity	> 100 ng/mL	(> 250 µmol/L)

Table 1 The above reference range was published in the Journal of Oncology Practice in 2010.<sup>3</sup>

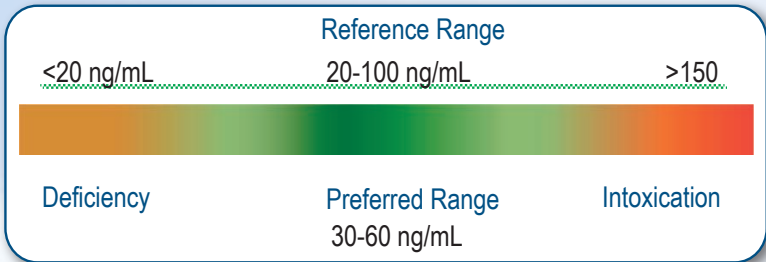


Table 2 The above reference range was published in the New England Journal of Medicine, 2007, and in American Journal of Clinical Nutrition, 2007.<sup>2</sup>

### 2.2 Prevalence of Vitamin D Deficiency

The incidence of Vitamin D deficiency (i.e, 25-(OH)D < 20 ng/mL) in US adults is approximately 25% for men and approximately 35% for women according to the data published in the American Journal of Clinical Nutrition in 2008.<sup>4</sup>

*“It has been estimated that 1 billion people worldwide do not reach the minimum optimal concentration of 30 ng/mL.”<sup>1</sup>*

### 3. Vitamin D: Beyond the bone

Vitamin D is well known for its classic role in the maintenance of bone mineral density. In conjunction with parathyroid hormone (PTH), Vitamin D plays a critical role in calcium homeostasis. However, in recent years, Vitamin D has been receiving increased attention due to the resurgence of Vitamin D deficiency and rickets in developed countries. The identification of extra-skeletal effects of Vitamin D also suggests unexpected benefits of Vitamin D in health and disease, extending beyond bone health. The possibility of extra-skeletal effects of Vitamin D was first noted with the discovery of the Vitamin D receptor (VDR)<sup>5-9</sup> in tissues and cells that are not involved in maintaining bone mineral homeostasis; including skin, placenta, pancreas, breast, prostate, colon cancer cells, and activated T cells. Numerous scientific and medical researchers have provided strong evidence supporting the correlations between Vitamin D insufficiency and increased risks of developing non-skeletal pathologies including cancer, heart diseases, stroke, diabetes, hypertension, autoimmune diseases, infectious diseases, and aging.<sup>10</sup> In 2013, Professor Sylvia Christakos, an expert in Vitamin D research at New Jersey Medical School, published a review article titled “Vitamin D Beyond Bone” in ANNALS OF THE NEW YORK ACADEMY OF SCIENCES<sup>10</sup>, where she summarized that Vitamin D deficiency and the increased risks of chronic diseases beyond bone health, as seen in Table 3.

1	Cancer	6	Diabetes
2	Heart diseases	7	Obesity
3	Autoimmune diseases	8	Muscle weakness
4	Lung function and pulmonary disease	9	Aging
5	Renal disease	10	Pregnancy

Table 3

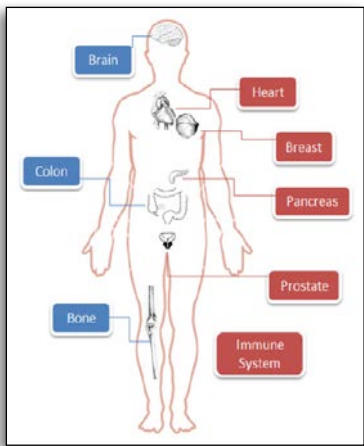


Figure 3

#### 3.1 Vitamin D & Vitamin D Receptor (VDR)

It is now recognized that Vitamin D exists in almost every tissue in the body, and that its effects are not limited to regulation of calcium and bone homeostasis. The discovery of the Vitamin D Receptor (VDR), by which Vitamin D exerts its action<sup>10</sup>, and recent epidemiological studies, has focused attention on a possible link between Vitamin D and a multitude of conditions including; cancer, heart disease, hypertension, diabetes, autoimmune diseases, infectious diseases and aging. Figure 3 depicts some of the major locations of VDR's in tissues and organs of the human body.

It is reported that approximately 3% of the human genome is either regulated directly or indirectly by the Vitamin D system.<sup>11-14</sup> This may explain why Vitamin D appears to play a role in many diseases, depicted in figures 4 and 5.<sup>10</sup>

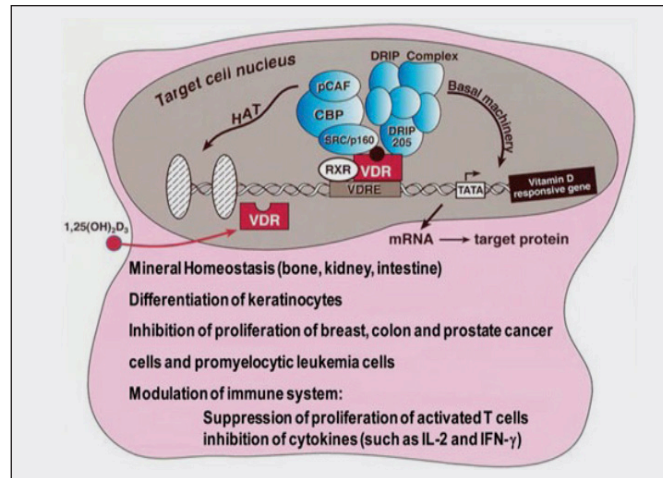


Figure 4

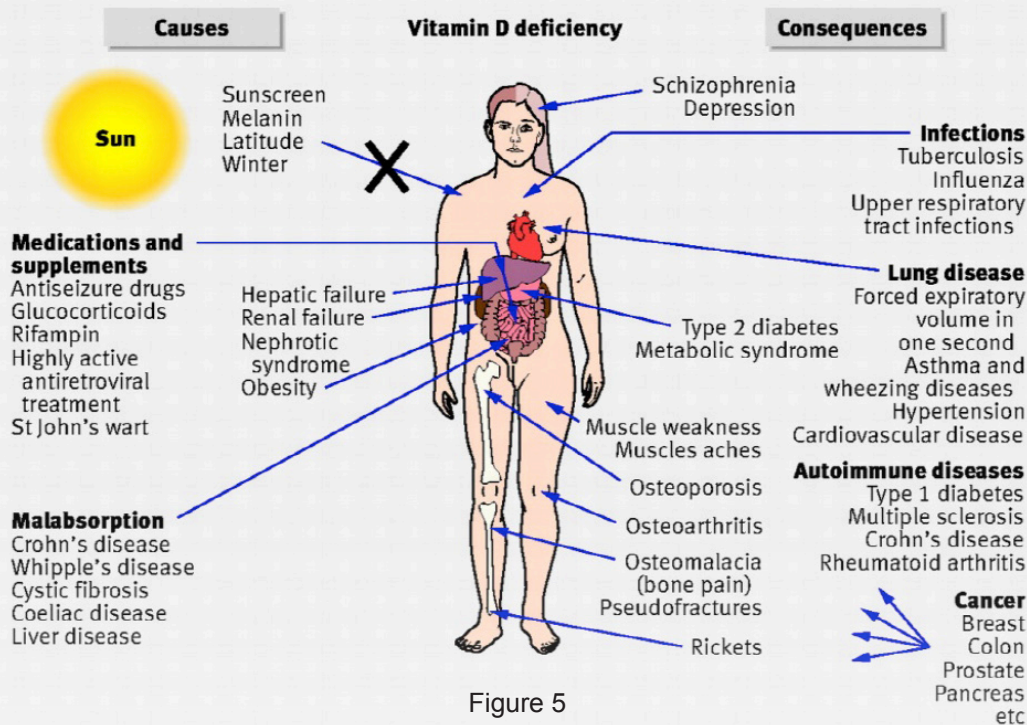


Figure 5

## 3.2 Representative Studies on Vitamin D Deficiency and Risk of Chronic Diseases

### (1) Vitamin D Deficiency and Cancer

Vitamin D deficiency is a critical factor in the pathology of at least 17 varieties of cancer.<sup>15-20</sup> A pooled analysis of two studies with 880 cases of breast cancer and 880 controls demonstrated that individuals with serum 25-(OH)D levels of approximately 52 ng/mL had 50% lower risk of breast cancer than those with levels <13 ng/mL.<sup>21</sup>

A four year trial including 1085 healthy women supplemented with either a placebo, calcium or calcium + Vitamin D showed that Vitamin D supplementation reduced by 77% the relative risk of developing cancer.<sup>22</sup>

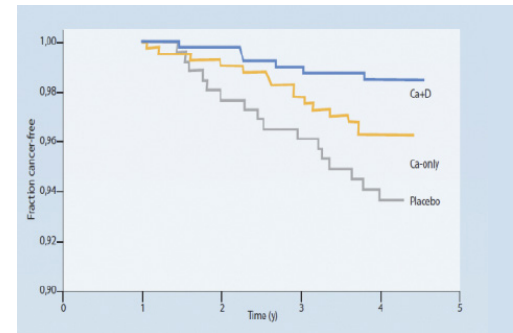


Figure 6

### (2) Vitamin D Deficiency and Heart Disease

A prospective study was done in which 1,739 participants without prior cardiovascular disease were followed-up for five years. Results showed that individuals with hypertension and 25-(OH)D levels <15 ng/mL had a 200% higher risk of cardiovascular events compared to those with levels >15 ng/mL.<sup>23</sup>

D levels <15 ng/mL had a 200% higher risk of cardiovascular events compared to those with levels >15 ng/mL.<sup>23</sup>

A seven year follow-up study of 3,258 patients referred for coronary angiography showed that decreasing 25-(OH)D levels (Q1=7.6 ng/mL, Q2=13.3 ng/mL, Q3=18.9 ng/mL, Q4=28.4 ng/mL) were associated with an increased risk for all-cause and cardiovascular mortality (Figure 7).<sup>24</sup>

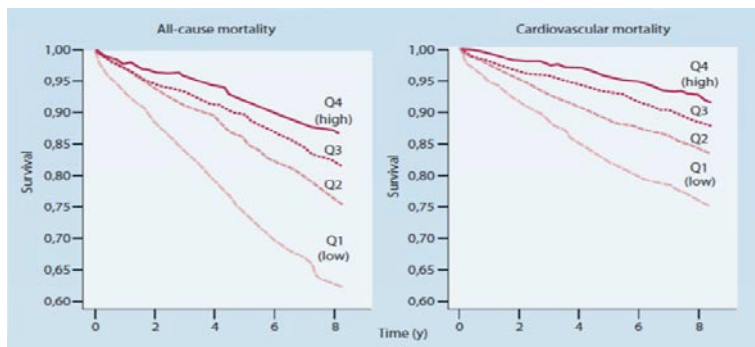


Figure 7

### (3) Vitamin D Deficiency and Diabetes

In a cohort of 10,366 children, Vitamin D supplementation with daily doses of 2,000 IU was associated with a 78% reduced risk of developing type 1 diabetes compared to lower doses.<sup>25</sup>

A ten year follow-up of 524 non-diabetic adults demonstrated an inverse association between baseline serum 25-(OH)D levels and future hyperglycemia and insulin resistance.<sup>26</sup>

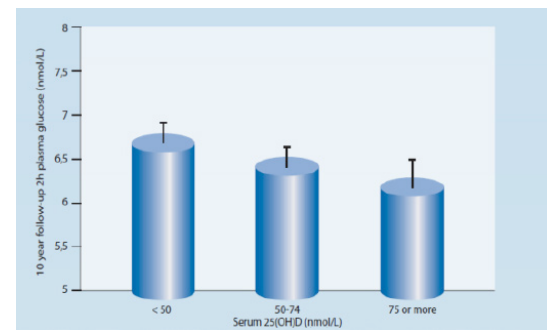


Figure 8



#### *(4) Vitamin D Deficiency and Autoimmune Disease*

The risk of multiple sclerosis in a white population of 148 patients and 296 controls was demonstrated to be 51% lower for individuals with 25(OH) Vitamin D levels >40 ng/mL compared to levels <30 ng/mL<sup>27</sup>. A study including 103 patients and 110 controls showed that for every 4 ng/mL increase of serum 25(OH) Vitamin D, the odds of multiple sclerosis were reduced by 19% in women.<sup>28</sup>

According to a report published online Jan. 20, 2014 in JAMA Neurology<sup>29</sup>, correcting Vitamin D deficiency early in the course of the disease is important. The lead researcher of the study, Dr. Alberto Ascherio, a professor of epidemiology and nutrition at the Harvard School of Public Health, stated that “These findings, combined with previous evidence that Vitamin D deficiency is a risk factor for MS, and the immunological effects of Vitamin D strongly suggest that maintaining an adequate Vitamin D status is important in the treatment of MS.”

#### *(5) Vitamin D Deficiency and Renal Disease*

Typically, chronic kidney disease (CKD) and end state renal disease (ESRD) patients have low levels of Vitamin D. The condition is correctable with the administration of Vitamin D supplements.<sup>23, 24</sup> In one study, correcting the deficiency decreased bone turnover, increased albumin levels, and brought more patients within the KDOQI guidelines for calcium and phosphorus.

In another three year prospective study of peritoneal dialysis patients, those with the lowest serum levels of Vitamin D had an increased risk of cardiovascular events.<sup>30-32</sup> However, the overall benefit of maintaining an adequate Vitamin D level in dialysis patients should be weighed against the potential risk of development of vascular calcification or a dynamic bone disease

#### *(6) Vitamin D Deficiency and Infectious Disease*

Deficiency of Vitamin D has long been implicated in activation of tuberculosis (TB).<sup>33</sup> Serum levels of Vitamin D in TB patients are lower than in healthy controls.<sup>34-35</sup> Paradoxically, prolonged treatment of TB also causes a decline in serum Vitamin D levels.<sup>34</sup> Several studies have suggested that Vitamin D is a potent immunomodulator of innate immune responses by acting as a cofactor for induction of anti-mycobacterial activity. A study conducted in Pakistan with a cohort of TB patients and their contacts (N=129) showed that 79% of TB patients are Vitamin D deficient. Low Vitamin D levels were associated with a 5-fold increased risk for the progression to TB.



## 4. Analytical Methods for Vitamin D Determination

Historically, Vitamin D was measured by competitive binding methods, high-performance liquid chromatography (HPLC), and radioimmunoassay (RIA) methods.<sup>36,37</sup>

A commonly used RIA kit, developed by DiaSorin S.p.A was the method used by many reference laboratories and was considered the gold standard in the past decades until recently LC-MS/MS method was developed. LC-MS/MS method has been used to establish reference ranges and NIST standard materials for Vitamin D, and has now been considered as the gold standard for Vitamin D assay's. However, these methods (HPLC, RIA, and LC-MS/MS) tend to be labor intensive and technically difficult. They are often not fully automated, and of low throughputs. LC-MS/MS method is not commonly available in clinical laboratories.

In the past few years, several FDA-approved, fully automated immunoassay methods for 25-hydroxy Vitamin D assay became available, including quantitative chemiluminescent immunoassay (CLIA) methods by DiaSorin, IDS, Siemens, Abbott and electrochemiluminescence (ECL) method by Roche. These immunoassay methods are solid phase (magnetic beads) based heterogeneous immunoassays involving multiple phase separation steps (washing steps), and requiring special instruments or manufacturer specific instruments to perform. Qualities of these immunoassays vary among manufacturers in terms of assay sensitivity and specificity. Some of these immunoassays do not equally measure 25-hydroxy Vitamin D2 and D3, and some have poor precision and correlation in the low end, some have poor traceability to NIST standard material.<sup>38</sup>

Recently, Diazyme Laboratories developed an innovative 25-hydroxy vitamin D assay that is a homogenous enzyme-immunoassay for use on general clinical chemistry analyzers, the instruments that are most commonly available in clinical laboratories. This is the first fully automated Vitamin D assay for clinical chemistry analyzers. The assay utilizes Diazyme's FemtoQuant™ technology that ensures the high sensitivity and specificity of assay without phase separation steps. The assay is FDA-approved, CE marked, and the results correlate well with LC-MS/MS and DiaSorin Liaison methods. This new Vitamin D assay allows many clinical laboratories to run the total Vitamin D test in house, at a cost effective price, without the need for special instrumentation.



# 4.1 Diazyme Enzyme-Immunoassay FemtoQuant™ Total 25-Hydroxy Vitamin D Assay\*

## (1) Assay Principle

Diazyme’s Enzyme-Immunoassay FemtoQuant™ 25-Hydroxy Vitamin D assay is based on the principle of  $\alpha$ -complementation of the enzyme  $\beta$ -galactosidase and the competition between an enzyme donor-25-(OH)D conjugate, the 25-(OH)D in a serum sample, and an anti-Vitamin D antibody. Samples with higher 25-(OH)D concentrations produce higher  $\beta$ -galactosidase activities. A nitro-phenyl- $\beta$ -galactoside analogue (NPGA) is used as the enzyme substrate and the accumulation of the reaction product has a maximum absorbance at 415 nm. The 25-(OH)D concentration of a patient sample is proportional to the measured  $\beta$ -galactosidase activity.

## (2) Kit Configuration

Diazyme’s Vitamin D assay kit contains 1 sample diluent and 3 liquid stable reagents and calibrator set. The intended use is for the quantitative determination of 25-(OH)D levels in serum or plasma, on automated chemistry analyzers, and for the assessment of Vitamin D sufficiency.



Figure 9

## (3) Assay Procedure

The assay procedure for the Roche Modular P chemistry analyzer is shown below: Specimen calibrator, control and samples are first diluted on-board: 20  $\mu$ L of serum which is then diluted with 155  $\mu$ L of diluent. Once diluted an additional 20  $\mu$ L of the diluted specimen is then used for analysis.

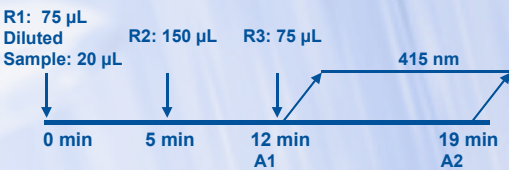


Figure 10

\* The Diazyme 25-OH Vitamin D Assay is intended for use in clinical laboratories for the quantitative determination of total 25-OH Vitamin D in human serum and plasma on automated chemistry analyzer. Measurement of 25-hydroxy Vitamin D (25-OH Vitamin D) is for the assessment of Vitamin D sufficiency.



#### (4) Assay Performance Summary

Diazyme's Vitamin D assay demonstrated high assay precision with total CV% ranging from 2.9% - 14% depending on the concentration of Vitamin D in the samples. The assay sensitivity gives an LOD value of 3.5 ng/mL and an LOQ value of 7.6 ng/mL. The assay is not affected by endogenous interfering substances including; bilirubin (40mg/dL), hemoglobin (100mg/dL), ascorbic acid (10 mM), and triglycerides (750 mg/dL). The assay measures 25-(OH) D3 and 25-(OH) D2 equally, providing accurate results for total Vitamin D concentrations.

The assay demonstrated excellent correlations with results determined by LC-MS/MS and DiaSorin (Figure 11) CLIA methods with  $R^2$  values of >0.95 and 0.96. The assay is traceable to NIST standard material (SRM 972) and has a wide linear range of 7.6 - 147.8 ng/mL (Figure 12).

The assay is fully automated, and can be run on clinical chemistry analyzers that are capable of taking 3 reagents and allowing for a total reaction time of 15 min or greater. The throughput of the assay on a Roche Modular P is > 100 tests/hour.

All reagents are liquid stable, and the on-board stability of the reagent is greater than four weeks. The performance characteristics of Diazyme Enzyme-Immunoassay FemtoQuant™ Vitamin D assay is summarized in the table on the following page.

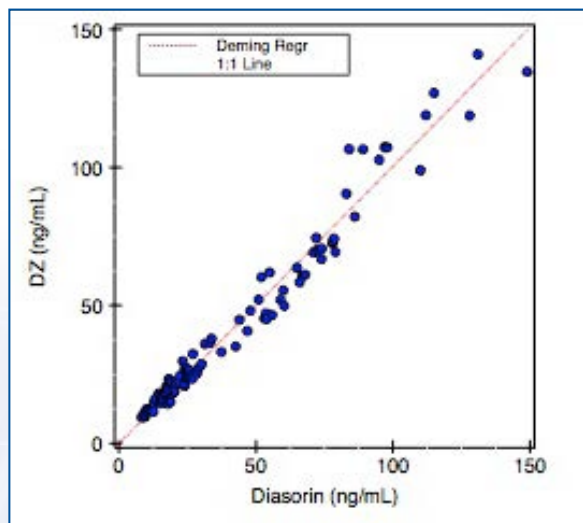


Figure 11

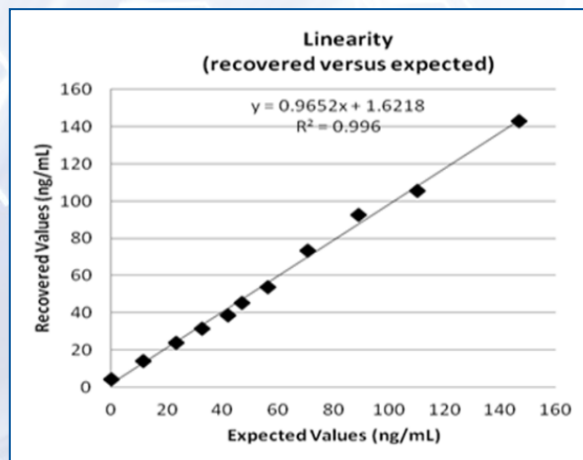




Figure 12



	<b>Diazyme FemtoQuant™ Vitamin D Assay</b>
<b>Method</b>	<b>Enzyme-Immunoassay</b>
<b>Instrument</b>	<b>General Chemistry Analyzers</b>
<b>Precision %CV:</b>	<b>2.9 - 14.0% 41 ng/mL: 4.1%; 93 ng/mL: 3.7%</b>
<b>Accuracy</b>	<b>R<sup>2</sup> = 0.984 against DiaSorin</b>
<b>Sensitivity</b>	<b>LoB: 2.0ng/mL; LoD: 3.5ng/mL; LoQ: 7.6 ng/mL</b>
<b>Measuring Range</b>	<b>7.6 - 147.8 ng/mL</b>
<b>Time to First Result</b>	<b>15 - 19 min Analyzer Dependent</b>
<b>Throughput</b>	<b>Roche Modular P: &gt;100 tests/hr. Ace Alera: &gt;50 tests/hr. Pentra 400: &gt;56 tests/hr.</b>
<b>Fully Automated</b>	<b>Yes</b>
<b>D2 and D3 Equal Accuracy</b>	<b>Yes</b>
<b>Directly Traceable NIST SRM 972</b>	<b>Yes</b>
<b>Regulatory Approvals</b>	<b>510(k) Cleared EU:  </b>

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## NOTES





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