

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, Inc., an affiliate of General Atomics, is 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.

Contents

SECTION I

1. What is Vitamin D?	3
1.1 Vitamin D Molecules and Metabolism	
2. Vitamin D Deficiency	4
2.1 Vitamin D Reference Range of Optimal Concentration	
2.2 Prevalence of Vitamin D Deficiency	
3. Vitamin D: Beyond the Bone	5
3.1 Vitamin D and Vitamin D Receptor (VDR)	
3.2 List of Vitamin D Deficiency and Risk of Chronic Diseases	
(1) <i>Vitamin D Deficiency and Cancer</i>	
(2) <i>Vitamin D Deficiency and Heart Diseases</i>	
(3) <i>Vitamin D Deficiency and Diabetes</i>	
(4) <i>Vitamin D Deficiency and Autoimmune Disease</i>	
(5) <i>Vitamin D Deficiency and Renal Disease</i>	
(6) <i>Vitamin D Deficiency and Infectious Disease</i>	
4. Analytical Methods for Vitamin D Determination	9
5. References	10

SECTION II

6. Diazyme EZ Vitamin D Assay	11
6.1 Assay Principle	
6.2 Kit Configuration	
6.3 Intended Use	
6.4 Assay Procedure	
6.5 Assay Performance Summary	

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)₂D. Of the major circulating forms, 25-OH Vitamin D reflects the levels of Vitamin D in the body.

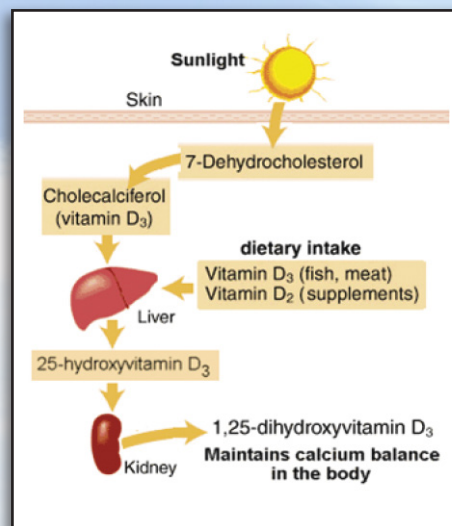


Figure 1

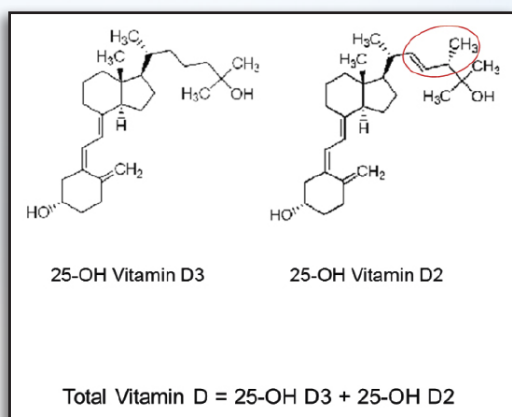


Figure 2

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)¹ and in the American Journal of Clinical Nutrition (J. Hathcock, 2007)², 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 2010.³

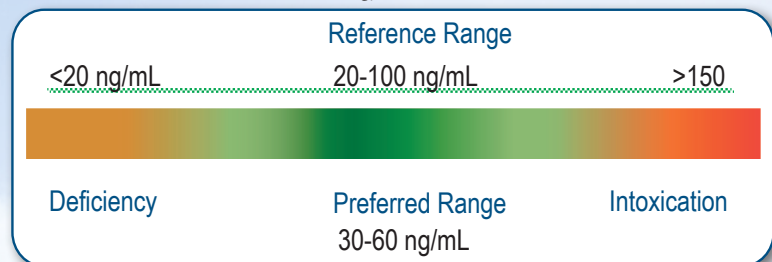


Table 2 The above reference range was published in the New England Journal of Medicine, 2007, and in American Journal of Clinical Nutrition, 2007.²

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 2008.⁴

*"It has been estimated that 1 billion people worldwide do not reach the minimum optimal concentration of 30 ng/mL."*¹

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)⁵⁻⁹ 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.¹⁰ 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 SCIENCES¹⁰, 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

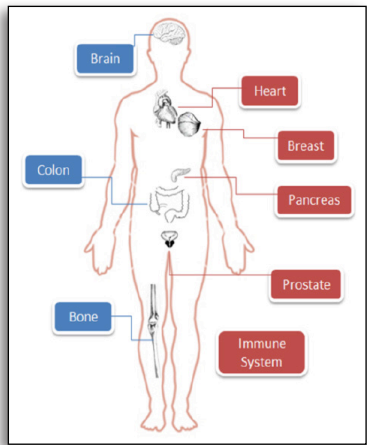


Figure 3

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 action¹⁰, 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.¹¹⁻¹⁴ This may explain why Vitamin D appears to play a role in many diseases, depicted in figures 4 and 5.¹⁰

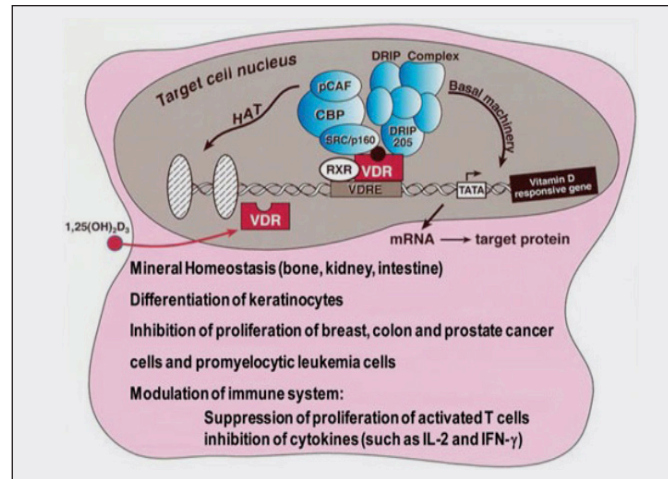


Figure 4

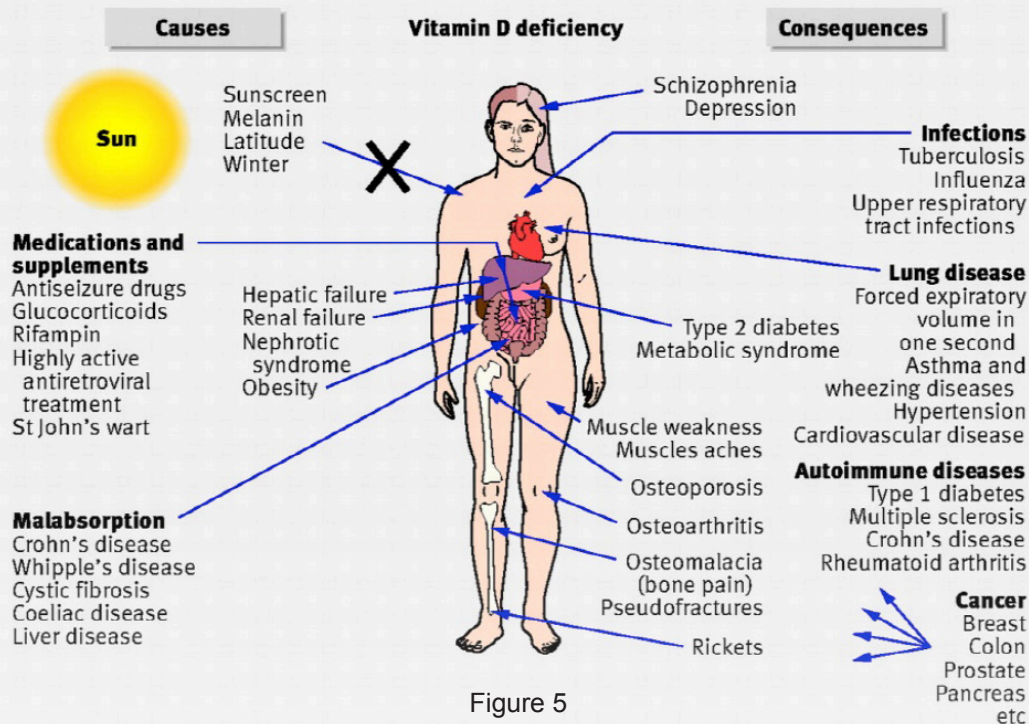


Figure 5

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.¹⁵⁻²⁰ 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.²¹

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.²²

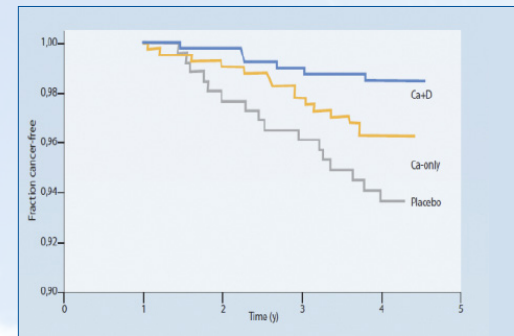


Figure 6

(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.²³

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).²⁴

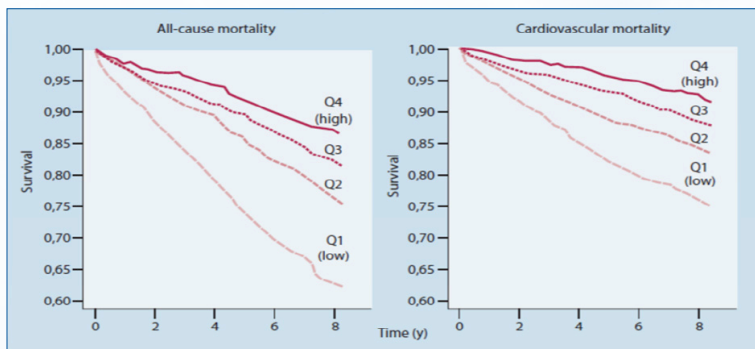


Figure 7

(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.²⁵

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.²⁶

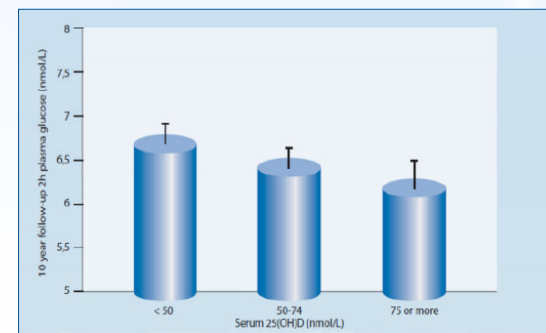


Figure 8

(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/mL²⁷. 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.²⁸

According to a report published online Jan. 20, 2014 in JAMA Neurology²⁹, 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.^{23,24} 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.³⁰⁻³² 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).³³ Serum levels of Vitamin D in TB patients are lower than in healthy controls.³⁴⁻³⁵ Paradoxically, prolonged treatment of TB also causes a decline in serum Vitamin D levels.³⁴ 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

Traditionally, analytical methods for Vitamin D determination have been measured by competitive binding, high-performance liquid chromatography (HPLC), and radioimmunoassay (RIA) methods^{36,37}. With the development of the LC-MS/MS method, Vitamin D reference ranges and NIST standard materials have shifted to what most view as the new gold standard.

However, despite their functionality, these methods (HPLC, RIA, and LC-MS/MS) tend to be labor intensive and technically difficult. They are often not fully automated, have low throughputs, and LC-MS/MS method is not commonly available in most clinical laboratories. In the past few years, the FDA has approved several fully automated immunoassay methods for 25-(OH)D. These immunoassay methods are solid phase (magnetic beads) based heterogeneous immunoassays, involving multiple phase separation steps (washing steps), and require 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 measure 25-hydroxy Vitamin D2 and D3 equally, have poor precision and correlation at the low end, and have poor traceability to NIST standard reference material.³⁸

In January of 2018, Diazyme Laboratories, Inc. developed an innovative EZ Vitamin D assay that is the first and only 2-reagent, latex enhanced immunoturbidimetric assay for use on general clinical chemistry analyzers. The EZ Vitamin D assay is fully automated requiring no sample pre-treatment or pre-dilution steps. Diazyme's EZ Vitamin D assay ensures excellent assay throughput, precision and a wide assay dynamic range. Diazyme's EZ Vitamin D assay for clinical chemistry analyzers is FDA-cleared, CE marked, and the results correlate well with the LC-MS/MS method. This new Vitamin D assay allows many clinical laboratories to run the EZ Vitamin D test in house, at a cost effective price, without the need for special instrumentation.



5. References

1. M. Holick., *Vitamin D Deficiency.*, NEJM, 357:3; 266-81, 2007
2. J. Hathcock et al., *Risk Assessment for Vitamin D.*, Am J Clin Nutr, 85:6; 2007
3. Qamar J, Khan MD, and Fabian, C., *How I Treat Vitamin D Deficiency.*, Journal of Oncology Practice, 6:2; 97-101, 2010
4. Yetley EA., *Assessing the Vitamin D Status of the US population.*, Am J Clin Nutr, 88:558S-564S; 2008
5. Haussler MR., & McCain TA., *Basic and Clinical Concepts Related to Vitamin D Metabolism and Action.*, New Engl Jour Med, 297: 974-978, 1041-1050, 1977
6. Pike JW., *Intracellular Receptors Mediate the Biologic Action of 1,25-Dihydroxyvitamin D3.*, Nutr, 43, 161-168, 1985
7. Haussler MR., *Vitamin D Receptors: Nature and Function.*, Annu Rev Nutr, 6: 527-562, 1986
8. Baker A et al., *Cloning and expression of full-length cDNA encoding human vitamin D receptor.*, Proc Nat Acad Sci USA, 85: 3294-3298, 1988
9. Costa EM, Hirst MA, and Feldman D., *Regulation of 1,25-dihydroxyvitamin D3 receptors by vitamin D analogs in cultured mammalian cells.*, Endocrinology, 117: 2203-2210, 1985
10. Sylvia Christakos et al., *Vitamin D: beyond bone.*, Ann N.Y. Acad Sci, 0077-8923
11. Wang Y, Becklund BR, DeLuca HF., *Identification of a highly specific and versatile vitamin D receptor antibody.*, Arch Biochem Biophys, 494: 166-177, 2010
12. Bouillon R, Carmeliet G, Verlinden L, et al., *Vitamin D and human health: lessons from vitamin D receptor null mice.*, Endocr Rev, 29: 726-776, 2008
13. Carlberg C and Seuter S., *A genomic perspective on vitamin D signaling.* Anticancer Res, 29: 3485-3493, 2009
14. Sun J., *Vitamin D and mucosal immune function.*, Curr Opin Gastroenterol, 26:6; 591-595, 2010
15. Adorini L and Penna G., *Control of autoimmune diseases by the vitamin D endocrine system.*, Nat Clin Pract Rheumatol, 4:8; 404-12, 2008
16. Campbell FC, Xu H, El-Tanani M, Crowe P, and Bingham V., *The yin and yang of vitamin D receptor (VDR) signaling in neoplastic progression: operational networks and tissue-specific growth control.*, Biochem Pharmacol, 79:1; 1-9, 2010
17. Cannell JJ, Hollis BW, Zasloff M, and Heaney RP., *Diagnosis and treatment of vitamin D deficiency.*, Expert Opin Pharmacother, 9:1; 107-18, 2008
18. Gocek E, Studzinski GP., *Vitamin D and differentiation in cancer.*, Crit Rev Clin Lab Sci, 46:4; 190-209, 2009
19. Grau MV, Baron JA, Sandler RS, Haile RW, Beach ML, Church TR, Heber DJ., *Vitamin D, calcium supplementation, and colorectal adenomas: results of a randomized trial.*, Natl Cancer Inst, 95:23; 1765-71, 2003
20. Wu S and Sun J., *Vitamin D, Vitamin D Receptor, and Macroautophagy in Inflammation and Infection.*, Discov Med, 11:59; 325-335, 2011
21. Holick MF., *Vitamin D and health: evolution, biologic functions, and recommended dietary intakes for vitamin D.*, Humana Press, 3-33, 2010
22. Garland CF et al., *Vitamin D and Prevention of Breast Cancer: pooled analysis.*, J. Steroid Biochem. Mol. Biol, M 103:3-5; 708-11, 2007
23. Lappe JM et al., *Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial.*, Am J Clin Nutr, 85: 1586-91, 2007
24. Dobnig H et al., *Independent association of low serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels with all-cause and cardiovascular mortality.* Arch Intern Med, 168:12; 1340-1349, 2008
25. Hypponen E et al., *Intake of Vitamin D and risk of type 1 diabetes: a birth-cohort study.*, Lancet, 358:9292; 1500-3, 2001
26. Forouhi NG et al., *Baseline serum 25-hydroxyvitamin D is predictive of future glycemic status and insulin resistance: the Medical Research Council Ely Prospective Study 1990-2000.*, Diabetes, 57:10; 2619-25, 2008
27. Munger KL et al., *Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis.*, J Am Med Assoc, 296:23; 2832-2838, 2006
28. Knigt J et al., *Higher levels of 25-hydroxyvitamin D are associated with a lower incidence of multiple sclerosis only in women.*, Mult Scler, 15:1; 9-15, 2009
29. Alberto Ascherio et al., *Vitamin D as an early predictor of multiple sclerosis activity and progression.* JAMA Neurol., 71:3; 306-14, 2014
30. Bhan I, Burnett-Bowie S, Ye J, Tonelli M, and Thadhani R., *Clinical Measures Identify Vitamin D Deficiency in Dialysis.*, Clin J Am Soc Nephrol, 5: 460-467, 2010
31. Gal-Moscovici A and Sprague S., *Role of Vitamin D Deficiency in Chronic Kidney Disease.*, Journal Of Bone And Mineral Research, 22:2; 2007
32. Chaudhary K, Sangha H and Khanna R., *Peritoneal Dialysis First: Rationale.*, The American Society of Nephrology, 6: 447-456, 2011
33. Rook GA., *The role of vitamin D in tuberculosis.* Am Rev Respir Dis., 138: 768-70, 1988
34. Davies PD, Brown RC and Woodhead JS., *Serum Concentrations of Vitamin D metabolites in untreated tuberculosis.* Thorax, 40: 187-90, 1985
35. Nnoaham KE, Clarke A., *Low serum vitamin D levels and tuberculosis: a systematic review and meta-analysis.*, Int J Epidemiol, 37: 113-9, 2008
36. Herrmann W, Obeid R, Herrmann M and Williams P., *Measurement of vitamin D.* In: Herrmann W, Obeid R, eds. *Vitamins in the prevention of human disease.* 1st ed. Berlin/New York: Walter de Gruyter GmbH & Co KG; 395-9, 2011
37. Hart GR, Furniss JL, Laurie D, Durham SK., *Measurement of vitamin D status: background, clinical use, and methodologies.*, Clin Lab, 52: 335-4, 2006
38. Herrmann, M., *The measurement of 25-hydroxy vitamin D – an analytical challenge.*, Clinical Chemistry and Laboratory Medicine. 50:11; 1873-1875

6. Diazyme EZ Vitamin D Assay*

6.1 Assay Principle

The Diazyme EZ Vitamin D Assay is a direct particle-enhanced immunoturbidimetric assay. The assay's proprietary reagents are designed to dissociate Vitamin D from Vitamin D binding proteins, found in serum or plasma specimens, while particles coated with anti-Vitamin D antibodies bind to the dissociated Vitamin D, thereby causing agglutination. This agglutination is detected as an absorbance change (700 nm), with the magnitude of the change being proportional to the quantity of total Vitamin D in the sample. Specimen concentrations are determined by interpolation from a 5-point calibration curve prepared from calibrators of known concentrations. (US Patent #: 20180031581)

6.2 Kit Configuration

Diazyme's EZ Vitamin D assay kit contains 2 liquid stable reagents. The intended use is the quantitative determination of 25-OH Vitamin D (Vitamin D) in human serum and plasma, using automated chemistry analyzers, and for the assessment of Vitamin D sufficiency.

6.3 Intended Use

The Diazyme EZ Vitamin D assay is intended for use in clinical laboratories for the quantitative determination of 25-OH Vitamin D (Vitamin D) in human serum and plasma, using automated chemistry analyzers. Measurement of Vitamin D is used for the assessment of the Vitamin D sufficiency. For in vitro diagnostic use only.*



Figure 9

6.4 Assay Procedure

The assay test scheme and the specific application parameters for the Beckman AU 680 analyzer are shown in Figure 10.



Figure 10

6.5 Assay Performance Summary

Diazyme's Vitamin D assay demonstrated high assay precision with total CV% ranging from 2.5% - 16.6% depending on the concentration of Vitamin D in the samples. The assay sensitivity gives an LOD value of 2.9 ng/mL and an LOQ value of 7.6 ng/mL. The assay is not affected by endogenous interfering substances including; free bilirubin (40 mg/dL), Conjugated bilirubin (40mg/dL), Hemoglobin (600mg/dL), Total protein (12.0g/dL), Triglycerides (1000mg/dL), and Rheumatoid Factor (RF) (200 IU/mL). The assay measures 25-(OH) D2 and 25-(OH) D3 equally, providing accurate results for total Vitamin D concentrations.

The assay demonstrated excellent correlations with results determined by LC-MS/MS (Figure 11) CLIA method with R^2 value of 0.9785. The assay is traceable to NIST standard material (SRM 972), recovers DEQAS 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 2 reagents and allowing for a total reaction time of 10 min. The throughput of the assay on a Beckman AU 680 is approximately 300 tests/hour.

All reagents are liquid stable, and the on-board stability of the reagent is approximately four weeks. The performance characteristics of Diazyme EZ Vitamin D assay is summarized in the table on the following page.

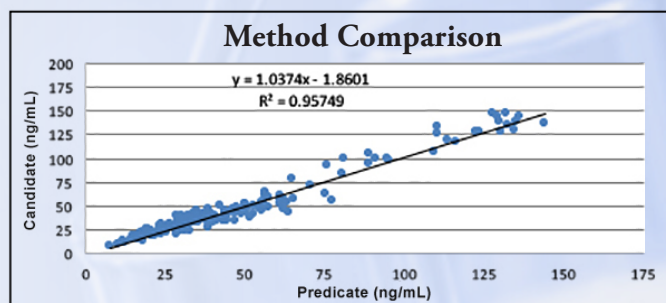


Figure 11

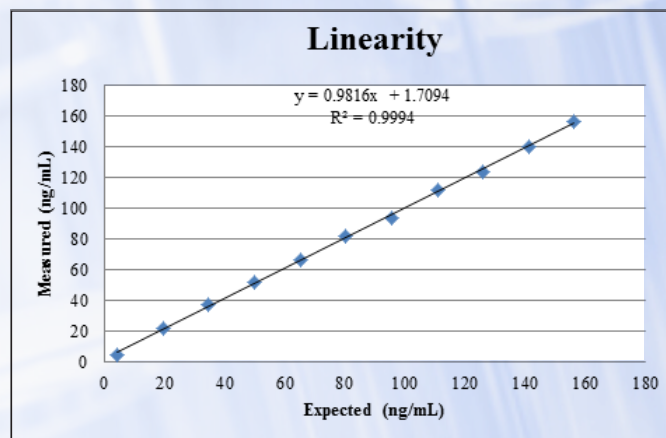



Figure 12

	Diazyme EZ Vitamin D Assay
Method	Latex Enhanced Immunoturbidimetric
Instrument	General Chemistry Analyzers
Precision % CV:	< 10%
Accuracy	$R^2 = 0.9785$ against predicate
Sensitivity	LoB: 1.2 ng/mL; LoD: 2.9 ng/mL; LoQ: 7.6 ng/mL
Measuring Range	7.6 - 147.8 ng/mL
Time to First Result	Approx. 10 min Analyzer Dependent
Throughput	AU 680: Approx 300 tests/hr.
Fully Automated	Yes
D2 and D3 Equal Accuracy	Yes
Directly Traceable NIST SRM 972	Yes
Regulatory Approvals	510(k) Cleared EU: CE 

NOTES





Diazyme Laboratories, Inc.
An Affiliate of General Atomics



Diazyme Laboratories, Inc.

Diazyme Europe GmbH

Zum Windkanal 21
01109 Dresden,
Deutschland

Tel: +49 (0) 351 886 3300
Fax: +49 (0) 351 886 3366

www.diazyme.com
sales@diazyme.com

12889 Gregg Court
Poway, CA 92064

Tel: 858-455-4768
888-DIAZYME

Fax: 858-455-3701

www.diazyme.com
sales@diazyme.com

Shanghai Diazyme Co., Ltd.

Room 201, 1011 Halei Road
Zhangjiang Hi-tech Park
Shanghai, 201203
People's Republic of China

Tel: 086-21-51320668
Fax: 086-21-51320663

www.lanyuanbio.com
service@lanyuanbio.com

