



US 20180031581A1

(19) **United States**(12) **Patent Application Publication**
YUAN et al.(10) **Pub. No.: US 2018/0031581 A1**(43) **Pub. Date: Feb. 1, 2018**(54) **METHODS AND KITS FOR ASSAYING A VITAMIN D MOIETY**(52) **U.S. Cl.**CPC **G01N 33/82** (2013.01); **A61K 31/593** (2013.01); **G01N 33/53** (2013.01)(71) Applicant: **Diazyme Laboratories, Inc.**, San Diego, CA (US)(72) Inventors: **Chong-Sheng YUAN**, San Diego, CA (US); **Fakhri Ben Habib SAIDA**, San Diego, CA (US)(73) Assignee: **Diazyme Laboratories, Inc.**, San Diego, CA (US)(21) Appl. No.: **15/663,578**(22) Filed: **Jul. 28, 2017****Related U.S. Application Data**

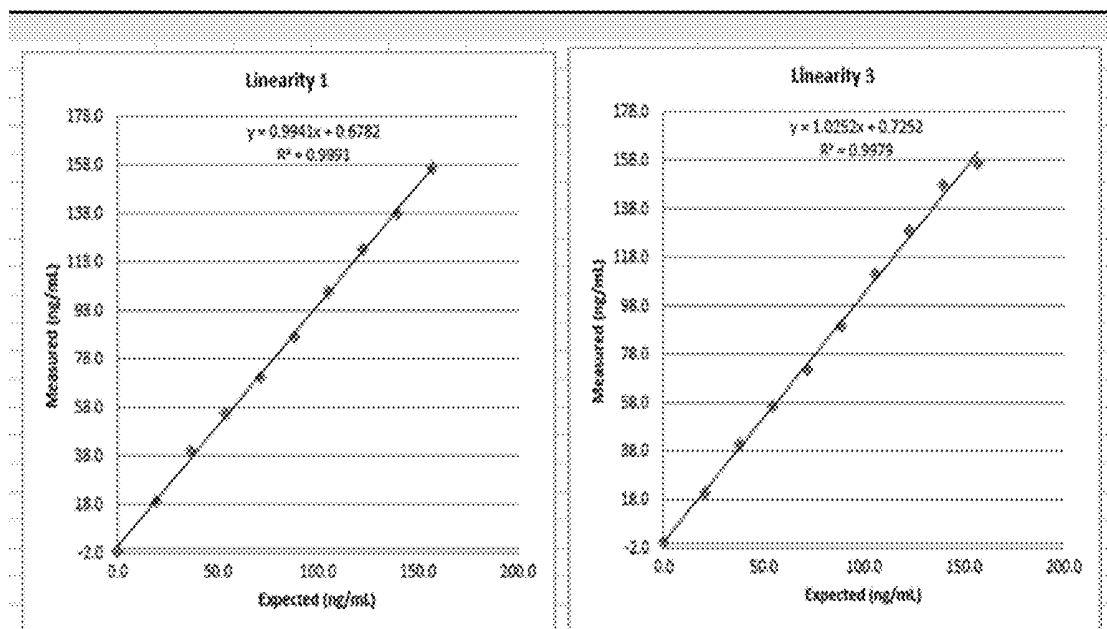
(60) Provisional application No. 62/368,940, filed on Jul. 29, 2016.

Publication Classification(51) **Int. Cl.****G01N 33/82** (2006.01)**G01N 33/53** (2006.01)**A61K 31/593** (2006.01)

(57)

ABSTRACT

The present invention provides methods and compositions, e.g., kits, for assaying a vitamin D moiety in a sample, comprising or using, inter alia, a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH, and at least two antibodies, e.g., at least two monoclonal antibodies, that are separately conjugated to particles, e.g., latex particles, wherein at least one of said antibodies (or the first antibody) has a specific binding affinity towards the vitamin D moiety, and at least another said antibody (or the second antibody) has a specific binding affinity towards the complex formed between the first antibody and the vitamin D moiety, if present in said sample. In some embodiments, the optical change due to the agglutination reaction between the antibodies and the vitamin D moiety is measured for determination of the amount of vitamin D content in the samples. Kits and reaction mixtures for assaying a vitamin D moiety in a sample are also provided.

**Assay linearity on AU 680 analyzer**

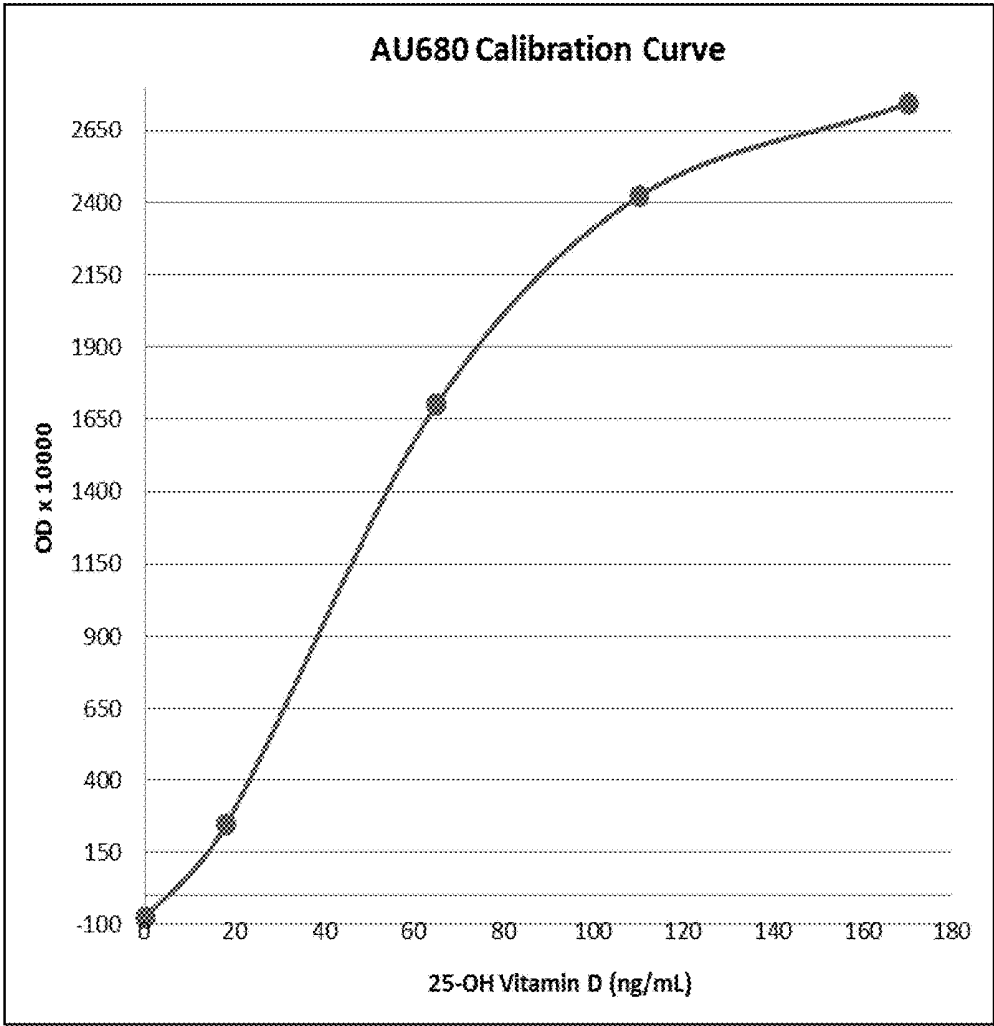


Figure 1. A typical calibration curve on AU680

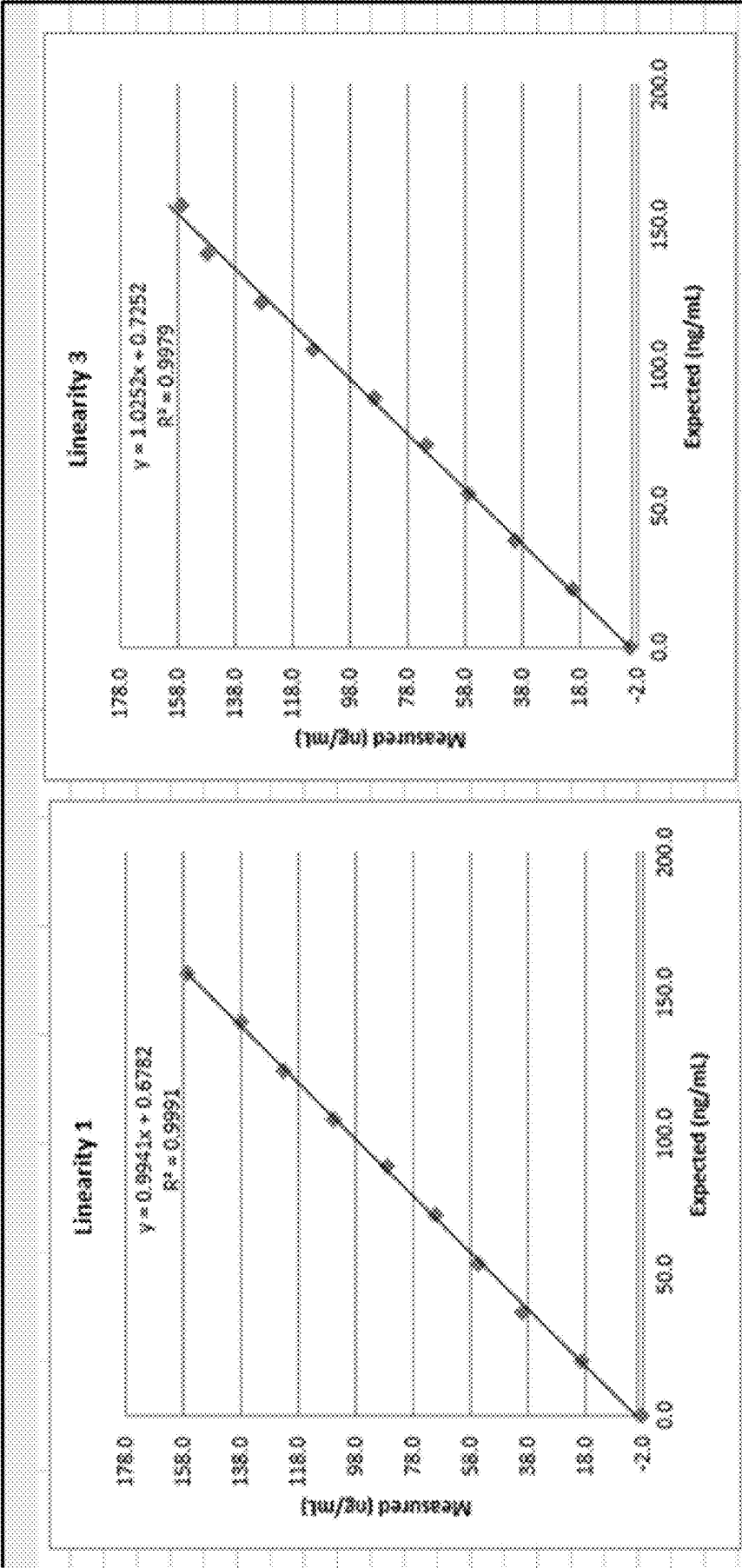


Figure 2. Assay linearity on AU 680 analyzer

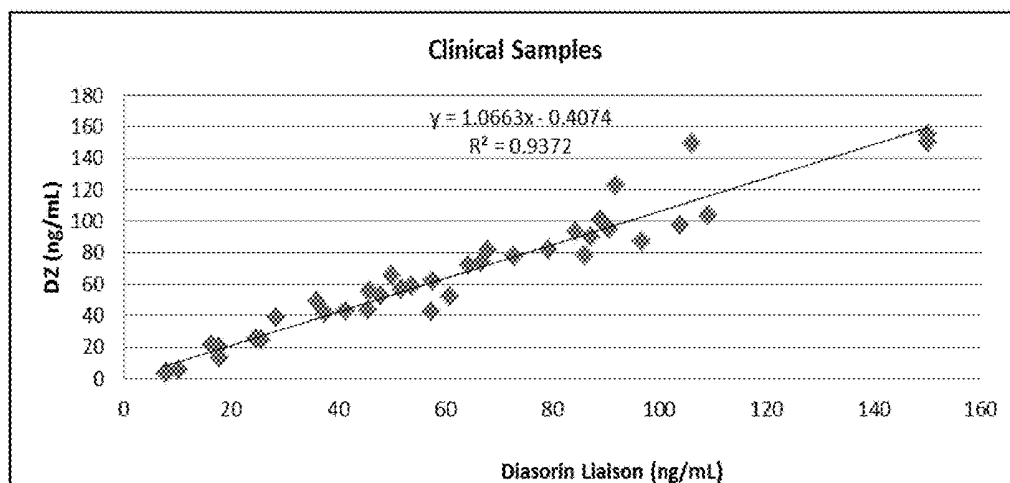


Figure 3. Assay accuracy in comparison with DiaSorin Liason method

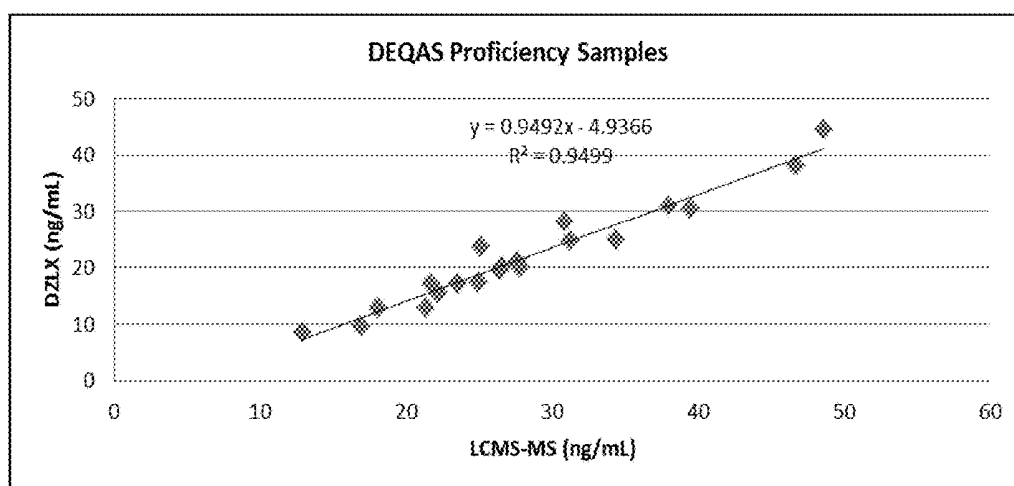


Figure 4. Assay accuracy in comparison with LC-MS/MS method

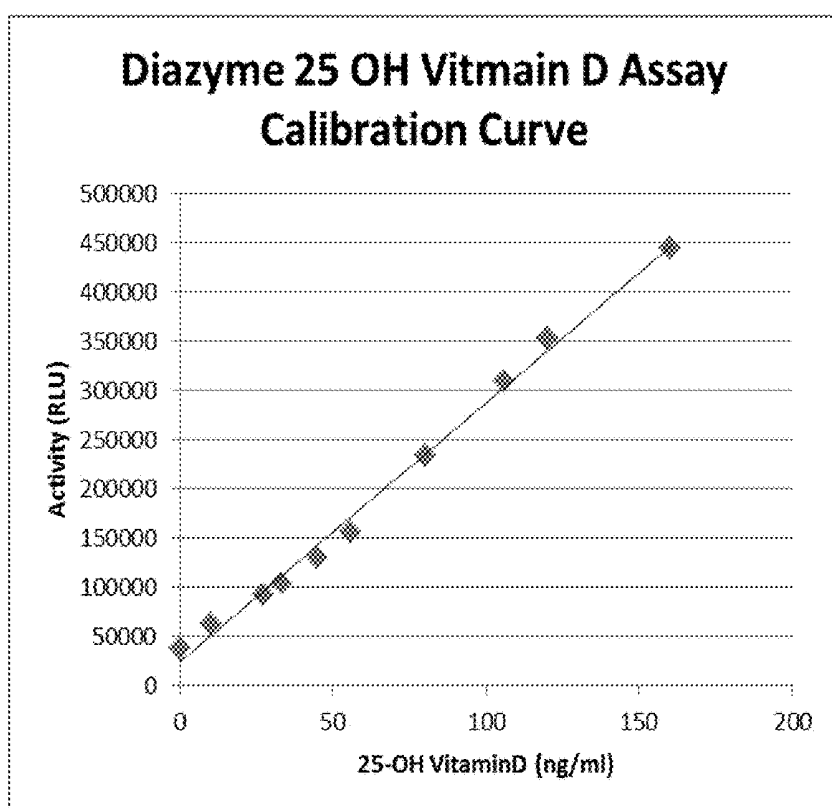


Figure 5. A typical calibration curve of chemiluminescent detection method

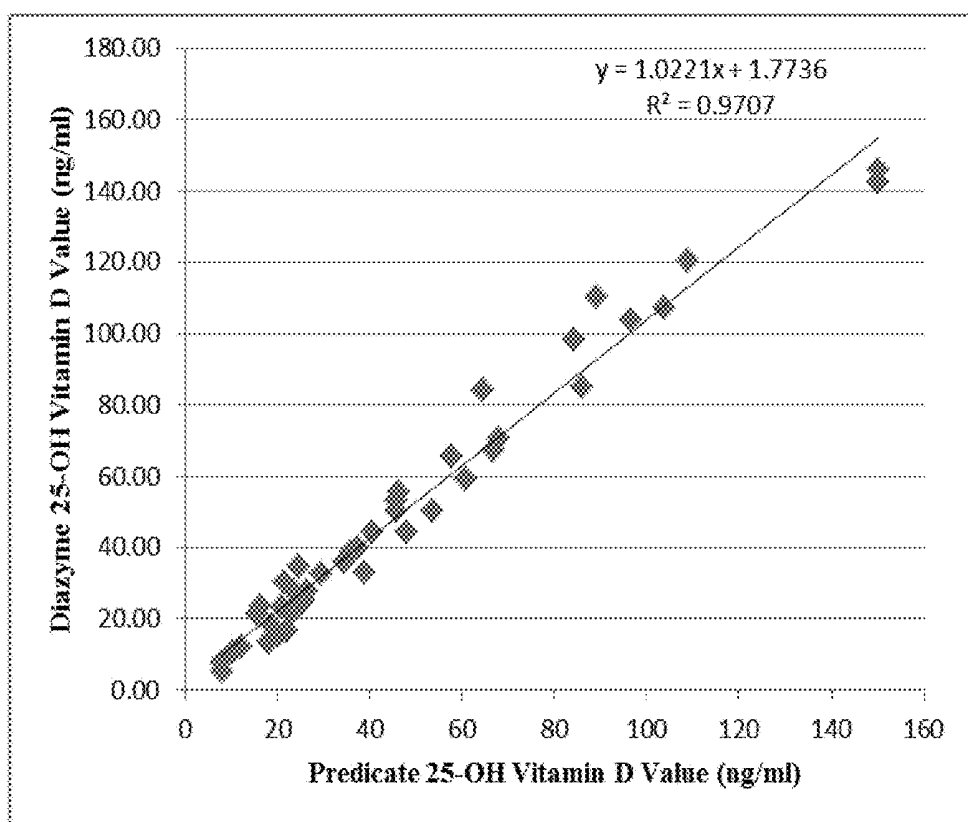


Figure 6. Method comparison data between the current invention method and a commercially available predicate method (immunoassay)

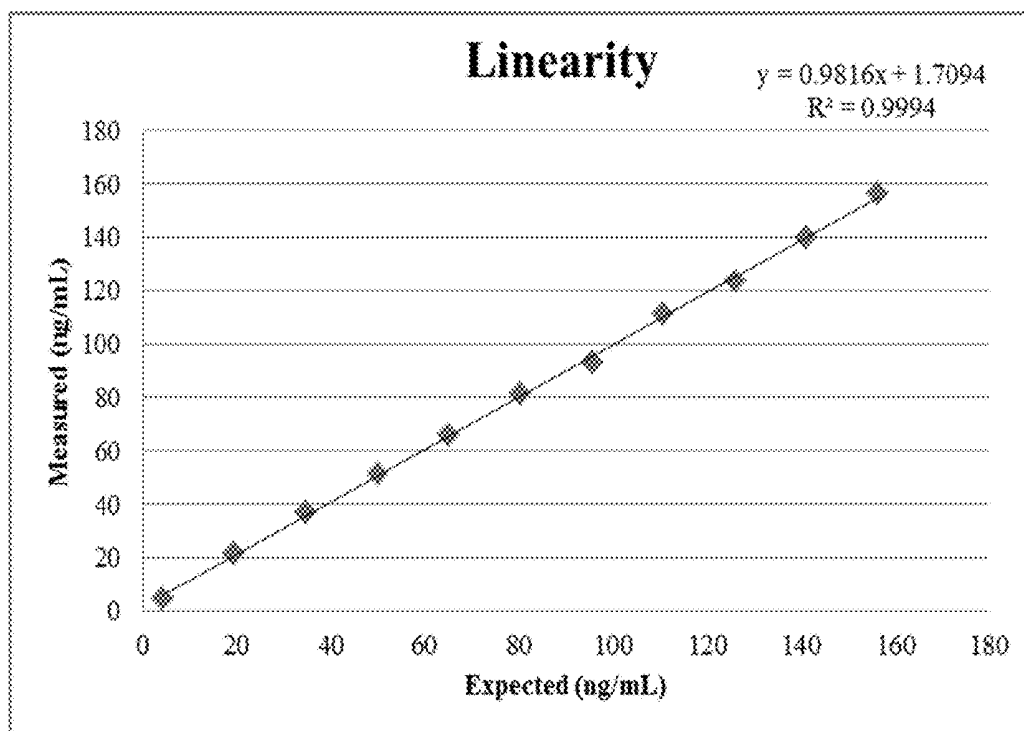


Figure 7. Linearity test results

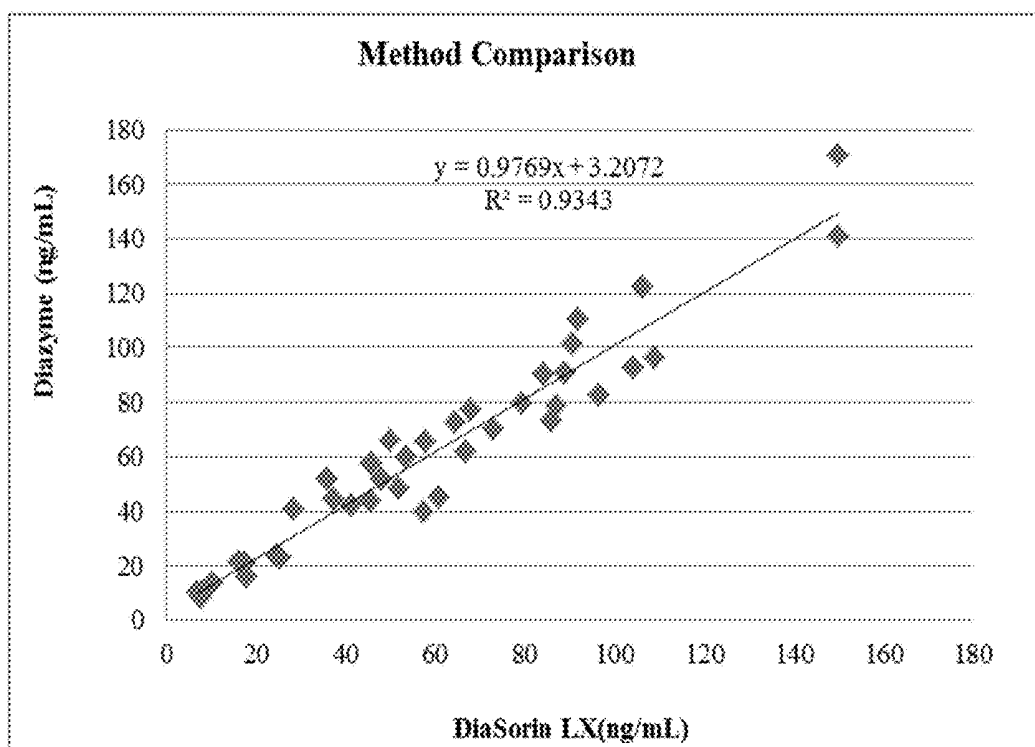


Figure 8. Method comparison results

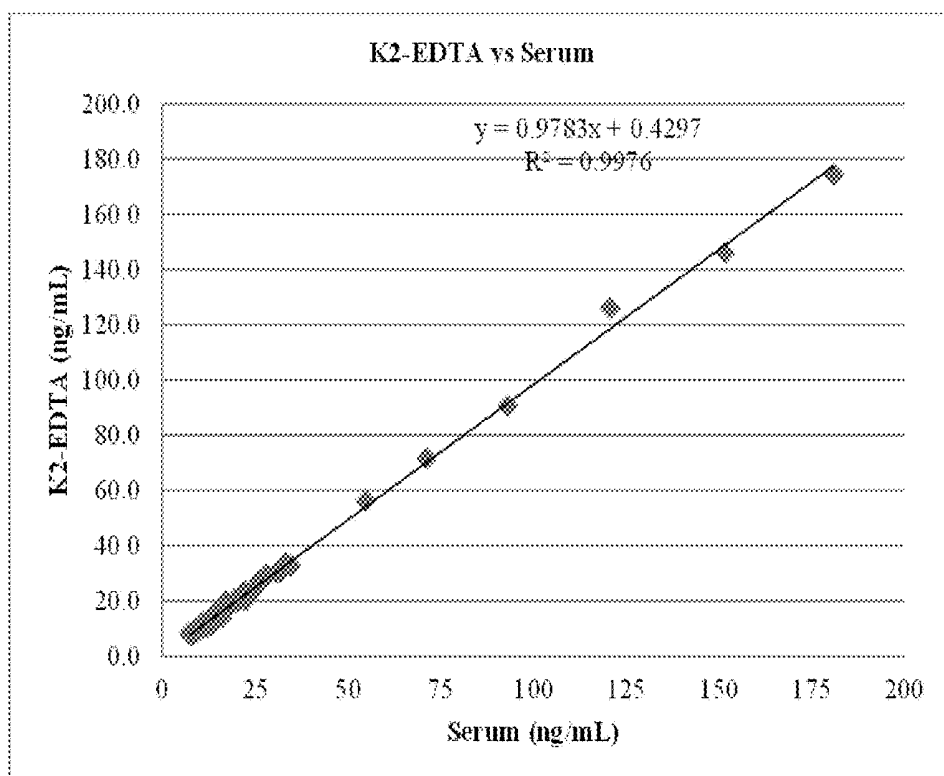


Figure 9. Matrix comparison results (K2-EDTA v. Serum)

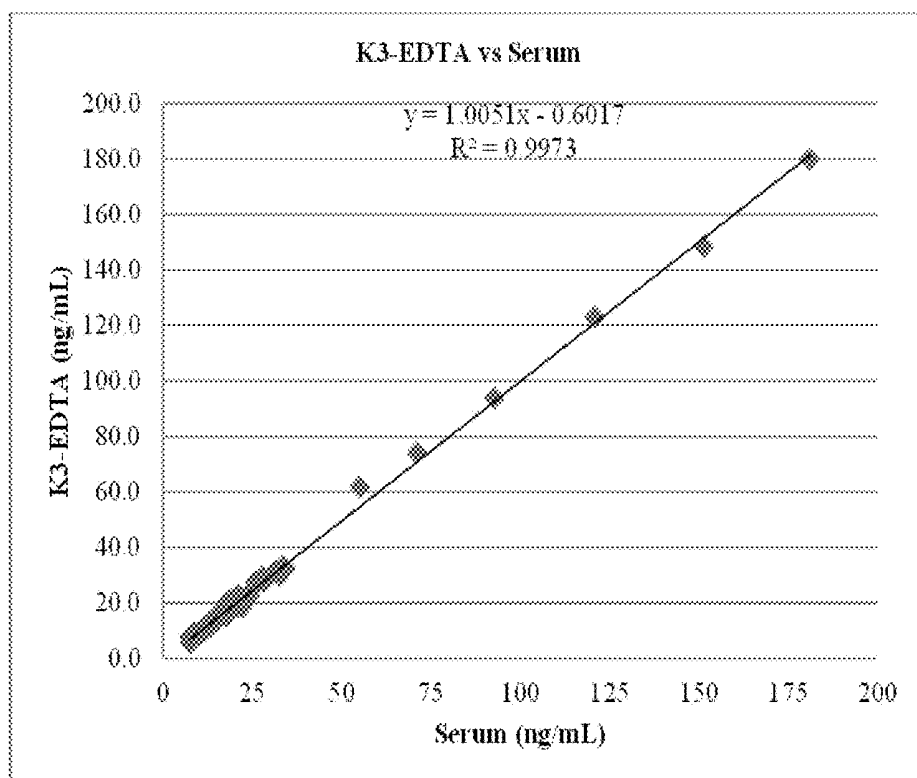


Figure 10. Matrix comparison results (K3-EDTA v. Serum)

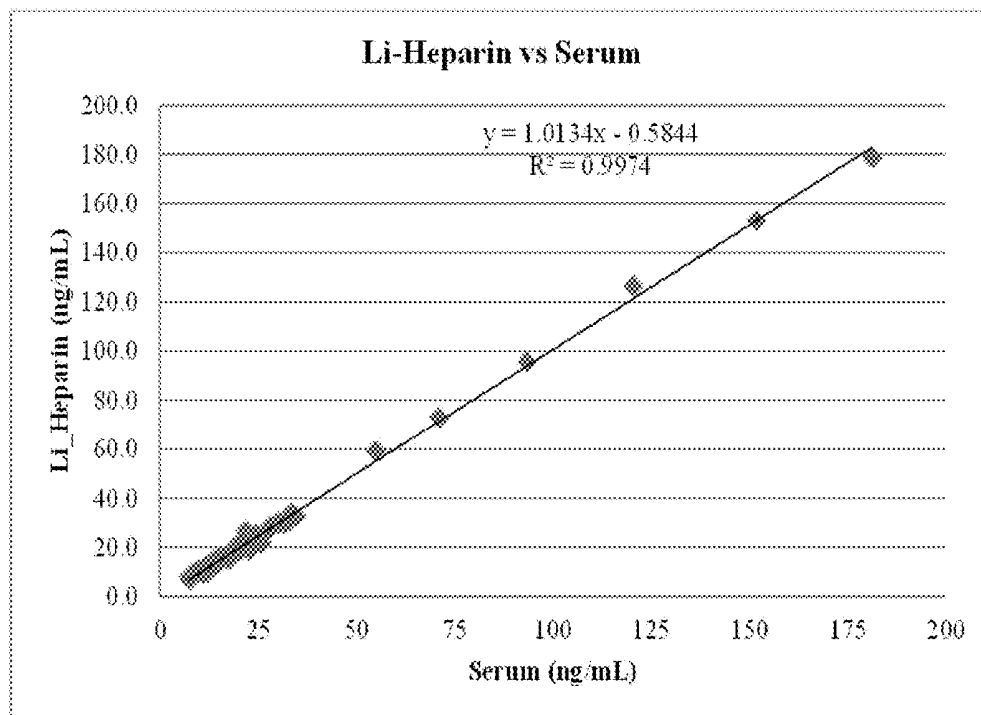


Figure 11. Matrix comparison results (K2-Li-Heparin v. Serum)

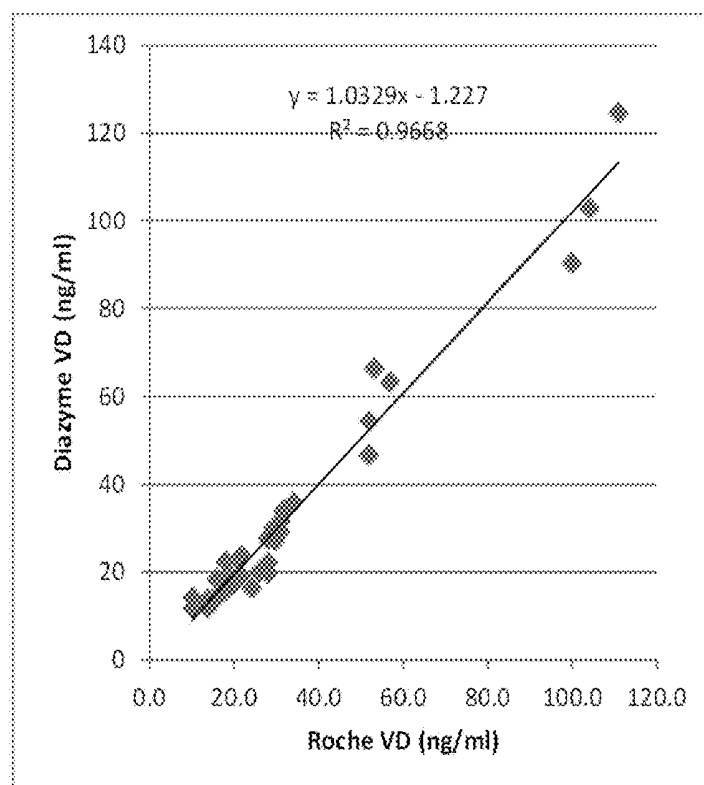


Figure 12. Method comparison results (v. Roche VD assay)

METHODS AND KITS FOR ASSAYING A VITAMIN D MOIETY

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the priority benefit of U.S. provisional application Ser. No. 62/368,940, filed Jul. 29, 2016, the content of which is incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] This invention generally relates to the field of vitamin D detection. In particular, the invention provides a novel methods and kits for assaying a vitamin D moiety in a sample such as a biological fluid.

BACKGROUND OF THE INVENTION

[0003] Vitamin D is a steroid-like, fat soluble prohormone. Vitamin D has two main forms: D2 (ergocalciferol) and D3 (cholecalciferol). Vitamin D3 can be manufactured by the body upon exposure to UV radiation. Both Vitamin D3 and Vitamin D2 are converted to the active hormone 1,25-dihydroxy Vitamin D through their metabolism in the liver and kidney.

[0004] Vitamin D3 is synthesized in 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 and the only prescription drug in US for Vitamin D deficiency is made of Vitamin D2. Vitamin D3 or D2 is metabolized by the liver to 25(OH)D, which is then converted by the kidneys to 1,25(OH)2D. 25(OH) Vitamin D is the major circulating form which reflects the levels of Vitamin D in the body, but 1,25(OH)2 Vitamin D is the most biologically active form.

[0005] Inadequate exposure to sunlight or low intake from diet or supplements may cause vitamin D deficiency. Vitamin D deficiency impairs bone mineralization, causing rickets in children and osteomalacia in adults and may contribute to osteoporosis. Recent studies have shown that Vitamin D deficiency is also linked to cancers, cardiovascular diseases, diabetes, multiple sclerosis, Parkinson disease, Alzheimer's disease, drug efficacy, and all-cause mortality.

[0006] A typical normal or sufficient range for Vitamin D is about 30-100 ng/mL. Vitamin D level at about 10-30 ng/mL is considered deficient. Vitamin D level less than 10 ng/mL is considered severely deficient. Vitamin D level more than 150 ng/mL is considered toxic.

[0007] Various vitamin D assays are known in the art. For example, various vitamin D assays are disclosed in U.S. Pat. Nos. 5,821,020, 7,087,395 B1, 7,482,162 B2, 7,964,363 B2, 8,133,694 B2, U.S. patent publication No. 2004/0132104 A1 and WO 2012/091569 A1.

[0008] There remains a need for a reliable, sensitive and specific method for assaying a vitamin D moiety in a sample such as a biological fluid, particularly one that can be conducted as a homogeneous assay and/or is amenable to automated clinical chemistry analyzers in typical clinical laboratory settings.

BRIEF SUMMARY OF THE INVENTION

[0009] In one aspect, the present invention provides a method for assaying a vitamin D moiety in a sample, which

method comprises: a) contacting a sample containing or suspected of containing a vitamin D moiety with a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH, and at least two antibodies, e.g., at least two monoclonal antibodies, that are attached to, e.g., separately attached to, surface(s) or particles, wherein one antibody, e.g., one monoclonal antibody or the first antibody, has a specific binding affinity towards the vitamin D moiety, and another antibody, e.g., another monoclonal antibody or the second antibody, has a specific binding affinity towards the complex formed between the first antibody and the vitamin D moiety; and b) assessing the binding between said specific antibodies and said vitamin D moiety to determine the presence, absence and/or amount of said vitamin D moiety in said sample. Optionally, the antibodies are different from a natural vitamin D binding protein for the vitamin D moiety.

[0010] In another aspect, the present invention provides a method for assaying a vitamin D moiety in a sample, which method comprises: a) contacting a sample containing or suspected of containing a vitamin D moiety with a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH, and at least two antibodies, e.g., at least two monoclonal antibodies, wherein at least one of said antibodies (or the first antibody) has a specific binding affinity towards the vitamin D moiety and is attached to a solid surface, e.g., solid surface of a microtiter plate or a particle, and at least another said antibody (or the second antibody) has a specific binding affinity towards the complex formed between said first antibody and said vitamin D moiety and is labelled with a signal generating moiety, and b) assessing binding between said antibodies, e.g., said specific antibodies, and said vitamin D moiety to determine the presence, absence and/or amount of said vitamin D moiety in said sample. Optionally, the antibodies are different from a natural vitamin D binding protein for the vitamin D moiety.

[0011] In still another aspect, the present invention provides a kit for assaying a vitamin D moiety in a sample, which kit comprise: a) a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH; b) at least two antibodies, e.g., at least two monoclonal antibodies, that are attached to, e.g., separately attached to, surfaces or particles such as latex particles, wherein one antibody, e.g., one monoclonal antibody or the first antibody has the affinity to bind with vitamin D moiety, and another antibody, e.g., another monoclonal antibody or the second antibody, has the affinity to bind with the complex formed between the first antibody and the vitamin D moiety.

[0012] In yet another aspect, the present invention provides a method for assaying a vitamin D moiety in a sample, which method comprises: a) contacting said sample with a first reagent comprising a buffer of acidic pH to dissociate said vitamin D moiety in said sample from its binding proteins; b) contacting said vitamin D moiety in said sample from step a) with a second reagent comprising particles, e.g., magnetic particles, coated with a first antibody that has a specific binding affinity to said vitamin D moiety to form a vitamin D moiety/first antibody complex; and c) contacting said vitamin D moiety/first antibody complex with a third reagent comprising a second antibody labelled with a signal generating molecule to form a complex among said vitamin D moiety, said first antibody complex and said second

antibody labelled with a signal generating moiety or molecule; and d) assessing said signal from said complex among said vitamin D moiety, said first antibody and said second antibody to determine the presence, absence and/or amount of the vitamin D moiety in said sample. In some embodiments, the signal generating moiety or molecule is selected from the group consisting of acridinium ester, isoluminol, alkaline phosphatase, horse radish peroxidase and fluorescein.

[0013] In yet another aspect, the present invention provides a reaction mixture for assaying a vitamin D moiety in a sample, which reaction mixture comprises: a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH; and at least two antibodies, e.g., at least two monoclonal antibodies, that are attached to, e.g., separately attached to, surface(s) or particles such as latex particles, wherein one antibody, e.g., one monoclonal antibody or the first antibody has the affinity to bind with vitamin D moiety, and another antibody, e.g., another monoclonal antibody or the second antibody, has the affinity to bind with the complex formed between the first antibody and the vitamin D moiety.

[0014] In some embodiments, the first antibody can be coated on surface(s) or particles such as magnetic particles, and another antibody or the second antibody can be labelled with signal generating molecules such as enzymes (e.g., alkaline phosphatase and peroxidase), or a chemiluminescent signal generating molecule such as acridinium ester, isoluminol, or a fluorescent molecule such as fluorescein and green fluorescent protein. In the presence of a vitamin D moiety in the reaction mixture, the second antibody binds to the complex formed between the vitamin D moiety from a sample and the first antibody coated on the surface(s) or particles to form a complex, e.g., a “sandwich” complex (first antibody-vitamin D-second antibody), and the amount of the “sandwich” formed on the surface(s) or particles is proportional to the amount of the vitamin D moiety in the sample, and can be quantitatively assessed by detecting the signal from the signal generating molecule that is labelled on the second antibody molecules.

[0015] In some embodiments, the at least two antibodies can be attached to a same particle or surface, or same type of particle(s) or surfaces. In other embodiments, the at least two antibodies can be attached to different particles or surfaces, or different types of particles or surfaces.

[0016] In some embodiments, the present methods and kits can use or incorporate selected elements or features of the methods and kits described and/or claimed in U.S. patent application Ser. Nos. 13/707,514 and 14/169,118, and PCT Application No. PCT/US2015/013831, which applications are incorporated by reference in their entireties.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] FIG. 1 illustrates an exemplary or a typical calibration curve of total 25(OH)D assay using the latex enhanced immunoassay method on an AU680 analyzer.

[0018] FIG. 2 illustrates an exemplary or a typical linearity curve of total 25(OH)D assay using latex particle enhanced immunoassay method on an AU680 analyzer.

[0019] FIG. 3 illustrates an exemplary or a typical method comparison data of total 25(OH)D assay using latex enhanced immunoassay method in comparison with a commercially available chemiluminescent immunoassay method (DiaSorin assay).

[0020] FIG. 4 illustrates an exemplary or a typical method comparison data of total 25(OH)D assay using latex particle enhanced immunoassay method in comparison with a gold standard method, the LC-MS/MS method.

[0021] FIG. 5 illustrates an exemplary or a typical calibration curve of total 25(OH)D assay using magnetic particle based immunoassay detection method.

[0022] FIG. 6 illustrates an exemplary or a typical method comparison data of total 25(OH)D assay using magnetic particle based immunoassay detection method in comparison with a commercially available chemiluminescent immunoassay method.

[0023] FIG. 7 illustrates exemplary linearity test results.

[0024] FIG. 8 illustrates exemplary method comparison results.

[0025] FIG. 9 illustrates exemplary matrix comparison results (K2-EDTA v. Serum).

[0026] FIG. 10 illustrates exemplary matrix comparison results (K3-EDTA v. Serum).

[0027] FIG. 11 illustrates exemplary matrix comparison results (K2-Li-Heparin v. Serum).

[0028] FIG. 12 illustrates exemplary method comparison results (v. Roche VD assay).

DETAILED DESCRIPTION OF THE INVENTION

[0029] For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

A. Definitions

[0030] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications referred to herein are incorporated by reference in their entireties. If a definition set forth in this section is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein incorporated by reference, the definition set forth in this section prevails over the definition that is incorporated herein by reference.

[0031] As used herein, “a” or “an” means “at least one” or “one or more.”

[0032] As used herein, “vitamin D moiety” refers to all members or forms of the Vitamin D family which is a group of fat-soluble secosteroids responsible for intestinal absorption of calcium and phosphate. Exemplary vitamin D forms include vitamin D₁, D₂, (ergocalciferol), D₃ (cholecalciferol), D₄, and D₅. Exemplary vitamin D moieties also include calcidiol, which is also known as calcifediol (INN), 25-hydroxycholecalciferol, or 25-hydroxyvitamin D-abbreviated 25(OH)D; and which is the specific vitamin D metabolite that is measured in serum to determine a person’s vitamin D status, and calcitriol, the biologically active form of vitamin D. In some embodiments, “vitamin D moiety” refers to 25-hydroxyvitamin D-abbreviated as 25(OH)D including D2 and D3 forms.

[0033] As used herein, “antibody” includes not only intact polyclonal or monoclonal antibodies, but also fragments thereof (such as Fab, Fab', F(ab')₂, Fv), single chain (ScFv), a diabody, a multi-specific antibody formed from antibody

fragments, mutants thereof, fusion proteins comprising an antibody portion, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity. An antibody includes an antibody of any class, such as IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. As used herein, the term “specifically binds” refers to the specificity of a binding reagent, e.g., an antibody, such that it preferentially binds to a defined analyte or target e.g., a vitamin D moiety. Recognition by a binding reagent or an antibody of a particular analyte or target in the presence of other potential targets is one characteristic of such binding. In some embodiments, a binding reagent that specifically binds to an analyte avoids binding to other interfering moiety or moieties in the sample to be tested.

[0034] A “vitamin D binding protein” is also known as gc-globulin (group-specific component). In some embodiments, a “vitamin D binding protein” refers to a Vitamin D-binding protein in the albumin family. A “vitamin D binding protein” is often found in plasma, ascitic fluid, cerebrospinal fluid and/or on the surface of many cell types. A “vitamin D binding protein” often binds to vitamin D and its metabolites and transports them to target tissues in vivo. An exemplary “vitamin D binding protein” in humans is encoded by the GC gene. In some embodiments, a “vitamin D binding protein” refers to a Vitamin D-binding protein known in the art, e.g., a vitamin D binding protein as described in the website: <https://en.wikipedia.org/wiki/Albumin>.

[0035] In some embodiments, “ascetic fluid” is used as described in the website: <https://en.wikipedia.org/wiki/Ascites>. In some embodiments, “cerebrospinal fluid” is used as described in the website: https://en.wikipedia.org/wiki/Cerebrospinal_fluid.

[0036] As used herein the term “assessing” is intended to include quantitative and qualitative determination in the sense of obtaining an absolute value for the amount or concentration of the analyte present in the sample, and also of obtaining an index, ratio, percentage, visual or other value indicative of the level of analyte in the sample. Assessment may be direct or indirect and the chemical species actually detected need not of course be the analyte itself but may for example be a derivative thereof or some further substance.

[0037] As used herein the term “sample” refers to anything which may contain an analyte for which an analyte assay is desired. The sample may be a biological sample, such as a biological fluid or a biological tissue. Examples of biological fluids include urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, amniotic fluid or the like. Biological tissues are aggregate of cells, usually of a particular kind together with their inter-cellular substance that form one of the structural materials of a human, animal, plant, bacterial, fungal or viral structure, including connective, epithelium, muscle and nerve tissues. Examples of biological tissues also include organs, tumors, lymph nodes, arteries and individual cell(s).

[0038] As used herein, “blood sample” refers to a whole blood sample or a plasma or serum fraction derived therefrom. Preferably, the blood sample refers to a human blood sample such as whole blood or a plasma or serum fraction derived therefrom. Also preferably, the blood sample is pre-treated before the assay by removing substantially all

hemoglobin (i.e., red blood cells) in order to eliminate or significantly reduce the oxidative interference from the hemoglobin molecules.

[0039] As used herein the term “whole blood” refers to a blood sample that has not been fractionated and contains both cellular and fluid components. As used herein, “whole blood” refers to freshly drawn blood which is tested before it clots, or a conventionally-drawn blood sample, which may be drawn into a vacutainer, and which may contain an anticoagulant, such as lithium-heparin, EDTA etc., or to which one or more other standard clinical agents may be added in the course of routine clinical testing.

[0040] As used herein, the phrase “substantially all hemoglobin has been removed” refers to a blood sample wherein preferably at least about 50%, 60% or 70%, more preferably, at least about 80%, 90% or 95%, and most preferably, at least about 96%, 97%, 98%, 99 or 100% of all hemoglobin-containing red blood cells in the sample have been removed to eliminate or significantly reduce the oxidative interference from hemoglobin.

[0041] As used herein, the term “plasma” refers to the fluid, non-cellular component of the whole blood. Depending on the separation method used, plasma may be completely free of cellular components, or may contain various amounts of platelets and/or a small amount of other cellular components. Because plasma includes various clotting factors such as fibrinogen, the term “plasma” is distinguished from “serum” as set forth below.

[0042] As used herein, the term “serum” refers to whole mammalian serum, such as whole human serum. Further, as used herein, “serum” refers to blood plasma from which clotting factors (e.g., fibrinogen) have been removed.

[0043] As used herein, the term “fluid” refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

[0044] As used herein, the term “disease” or “disorder” refers to a pathological condition in an organism resulting from, e.g., infection or genetic defect, and characterized by identifiable symptoms.

[0045] As used herein, “contacting” means bringing two or more components together. “Contacting” can be achieved by mixing all the components in a fluid or semi-fluid mixture. “Contacting” can also be achieved when one or more components are brought into contact with one or more other components on a solid surface such as a solid tissue section or a substrate.

[0046] As used herein, the term “comparing” generally means examining in order to note similarities or differences between two or more values. Preferably, “comparing” refers to quantitative comparisons such as, for example, subtracting one value from another, calculating a ratio of two values, calculating a percentage of one value with respect to another, or combining these types of calculations to produce a single number. As used herein, “comparing” further refers to comparisons made by a human, comparisons made by a computer or other processor, and comparisons made by a human in combination with a computer or other processor.

[0047] It is understood that aspects and embodiments of the invention described herein include “consisting” and/or “consisting essentially of” aspects and embodiments.

[0048] Other objects, advantages and features of the present invention will become apparent from the following specification taken in conjunction with the accompanying drawings.

B. Methods for Assaying a Vitamin D Moiety

[0049] In one aspect, the present invention provides a method for assaying a vitamin D moiety in a sample, which method comprises: a) contacting a sample containing or suspected of containing a vitamin D moiety with a buffer of acidic pH, at least two antibodies, e.g., at least two monoclonal antibodies, that are attached to, e.g., separately attached to, particles or surface(s), e.g., latex particles, one of the antibodies or the first antibody having a specific binding affinity towards said vitamin D moiety, and the another antibody or the second antibody having a specific binding affinity towards the complex formed between the first antibody and the said vitamin D moiety; and b) assessing binding (e.g., the degree of agglutination of particles due to formation of a complex formed among or between said at least two antibodies and said vitamin D moiety, e.g., a sandwich complex between said two monoclonal antibodies and said vitamin D moiety to determine the presence, absence and/or amount of said vitamin D moiety in said sample. Optionally, the antibodies are different from a natural vitamin D binding protein for the vitamin D moiety. In some embodiments, the at least two antibodies can be attached to a same particle or surface, or same type of particle(s) or surfaces. In other embodiments, the at least two antibodies can be attached to different particles or surfaces, or different types of particles or surfaces.

[0050] In another aspect, the present invention provides a method for assaying a vitamin D moiety in a sample, which method comprises: a) contacting a sample containing or suspected of containing a vitamin D moiety with a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH, and at least two antibodies, e.g., at least two monoclonal antibodies, wherein at least one of said antibodies (or the first antibody) has a specific binding affinity towards the vitamin D moiety and is attached to a solid surface, e.g., solid surface of a microtiter plate or a particle, and at least another said antibody (or the second antibody) has a specific binding affinity towards the complex formed between said first antibody and said vitamin D moiety and is labelled with a signal generating moiety, and b) assessing binding between said antibodies, e.g., said specific antibodies, and said vitamin D moiety to determine the presence, absence and/or amount of said vitamin D moiety in said sample. Optionally, the antibodies are different from a natural vitamin D binding protein for the vitamin D moiety.

[0051] In some embodiments, the present methods do not comprise a step of removing protein from the sample (See e.g., U.S. Pat. No. 5,821,020), such as the natural vitamin D binding protein for the vitamin D moiety, prior to assessing binding between the specific antibodies and the vitamin D moiety. In other embodiments, the present methods do not comprise any wash step.

[0052] The present methods can be conducted in any suitable assay format. In some embodiments, the present methods are conducted as a homogeneous assay. In other embodiments, the present methods are conducted as a heterogeneous assay.

[0053] A sample can be contacted with a buffer of acidic pH and two antibodies, e.g., two monoclonal antibodies, that are separately coated on particles, e.g., latex particles, in a single step, or in multiple steps, e.g., 2 or 3 steps. The exemplary multiple step contact order can be: 1) a buffer of acidic pH, incubating for a period of time before addition of the latex particles that are separately coated with the two said monoclonal antibodies; 2) a buffer of acidic pH, latex particles that coated with the first antibody, incubating for a period of time before addition of the latex particles coated with the said second antibody.

[0054] The pH in a final reaction mixture comprising the sample, the acid pH buffer, the particles, e.g., latex particles, separately coated with the two specific antibodies can be at any suitable value or range. In some embodiments, the pH in a final reaction mixture comprising the sample, the acidic pH buffer and the latex particles is at 2.5 or higher. In other embodiments, the pH in a final reaction mixture comprising the sample, the acidic pH buffer and the latex particles is at 13 or lower. In still other embodiments, the pH in a final reaction mixture comprising the sample, the acidic pH buffer and the latex particles is in a range from about 2.5 to about 13, e.g., at about 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, or 13.

[0055] In some embodiments, the present methods do not comprise a step of contacting the sample with a vitamin D releasing composition comprising a cyclodextrin, a sodium salicylate and NaOH, such as the vitamin D releasing composition disclosed in U.S. Pat. No. 7,087,395 B1.

[0056] In some embodiments, the present methods do not comprise a step of contacting the sample with a perfluoro alkyl acid, or a salt thereof, to release 25(OH) vitamin D from vitamin D binding protein, such as the step disclosed in WO 2012/091569 A1.

[0057] In some embodiments, the present methods do not comprise a step of contacting the sample with a serine protease with endo- and exoproteolytic activity to digest vitamin D binding proteins in the sample, such as the step disclosed in U.S. Pat. No. 7,964,363 B2.

[0058] The present methods can be used for any suitable purpose. In some embodiments, the present methods can be used to assess status of the vitamin D moiety in a subject, and the sample is a biological sample obtained and/or derived from the subject. The present methods can be used for assess status of the vitamin D moiety in any suitable subject, e.g., a mammal, a non-human mammal, a human or an experimental animal.

[0059] The present methods can be used for assaying a vitamin D moiety in any suitable sample. In some embodiments, the sample is a biological fluid, e.g., whole blood, plasma, serum or urine.

[0060] The present methods can be used for assaying any suitable vitamin D moiety in a sample. In some embodiments, the vitamin D moiety is vitamin D₃, vitamin D₂, a vitamin D metabolite or 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]. In other embodiments, the vitamin D moiety is 25-hydroxy-vitamin D (25(OH)D), e.g., 25(OH)D₃, 25(OH)D₂ or a sum of 25(OH)D₂ and 25(OH)D₃.

[0061] Any acidic pH buffer can be used in the present methods. In some embodiments, the acidic pH buffer can be sodium acetate, sodium citrate, or phosphoric acid, or other buffers that have buffer functions in the acidic pH range.

[0062] In other embodiments, the antibody specifically binds to 25(OH)D and the antibody specifically binds to the complex of 25(OH)D and the first antibody can be in any suitable forms. For example, a polyclonal antibody or a monoclonal antibody or fragments of the antibodies such as Fab fragment or a single chain antibody, can be used. In still other embodiments, exemplary antibodies disclosed in U.S. patent publication No. 2011/0097733 A1 can be used.

[0063] Any suitable particle can be used in the present particle based assay formats. In some embodiments, the particle comprises polystyrene, polymethyl methacrylate, polymethyl naphthalene, poly(divinylbenzene), polyvinyl naphthalene, co-polymer of styrene, acrylic acid divinylbenzene, naphthalene, carbon 60, magnetic beads, gold, silver, silica, silicon dioxide, chromium dioxide, and/or titanium dioxide. In other embodiments, the particle is a nanoparticle. The nanoparticle can have any suitable size or diameter. For example, the nanoparticle can have any diameter ranging from about 30 nm to about 500 nm, e.g., about 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 150 nm, 200 nm, 250 nm, 300 nm, 350 nm, 400 nm, 450 nm, or 500 nm.

[0064] The present methods can be used in any suitable assay format. In some embodiments, the present methods are conducted using a particle-enhanced immunoturbidimetric method. See e.g., U.S. Pat. No. 4,703,018. In other embodiments, the present methods are conducted using a particle-enhanced immunonephelometric method. See e.g., U.S. Pat. No. 4,690,906. In still other embodiments, the present methods are conducted using a magnetic particle (bead) based immunoassay method. See e.g., Yu et al., *Journal of Immunological Methods*, 218(1-2):1-8 (1998). In yet other embodiments, the present methods are conducted using a particle based immunoagglutination assay method. See e.g., Wang et al., *Clinical Chemistry*, 52(11):2065-2071 (2006).

[0065] In some embodiments, the size of magnetic particles can be ranged from 500 to 2000 nanometers, preferably from 800-1500 nanometers.

[0066] In some embodiments, the magnetic particles are conjugated with the first antibody, and the second antibody is conjugated with signaling molecules such as acridinium ester, isoluminol, alkaline phosphatase, or horse radish peroxidase.

[0067] In some embodiments, the vitamin D assay is performed using a two-step chemiluminescent immunoassay format. Samples are contacted with the acidic pH buffer, and followed by an incubation period of time before addition of an antibody conjugated on particles, e.g., magnetic particles. After incubation, the particles, e.g., magnetic particles, are washed 1-3 times with a washing buffer, and re-suspended in a buffer before addition of a second antibody labelled with a signaling molecule. After incubation, the magnetic particles are washed again, and re-suspended in a buffer before addition of a substrate needed for chemiluminescent reaction.

[0068] In some embodiments, the vitamin D assay is performed using a one-step chemiluminescent immunoassay format. Samples are contacted with the acidic pH buffer, the particles, e.g., magnetic particles, coated with the first antibody and the second antibody labelled with a signaling molecule at the same time. After an incubation period of time, the particles, e.g., magnetic particles, are washed 1-3 times and re-suspended in a buffer before addition of a substrate needed for the chemiluminescent reaction.

[0069] In some embodiments, the present methods are conducted using a homogeneous assay format. In other embodiments, the present methods are conducted using a heterogeneous assay format. In still other embodiments, the present methods are conducted using a sandwich or competitive assay format. In yet other embodiments, the present methods are conducted using a format of an enzyme-linked immunosorbent assay (ELISA).

[0070] Any suitable homogenous assay format can be used. In some embodiments, a homogenous assay is conducted in a single reaction mixture without phase separation or washing step, such as the particle separation and washing steps disclosed in U.S. patent publication No. U.S. 2004/0132104 A1 or U.S. Pat. No. 8,133,694 B2.

[0071] The present methods can be conducted with any suitable reaction time. In some embodiments, the present methods have a total assay time that is at about 60 minutes or shorter, e.g., about 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 3, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 minutes or 1 minute. For example, the present methods can have an assay time from initiation, e.g., addition of a sample and/or a reagent(s), to signal readout time, that is at about 60 minutes or shorter, e.g., about 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 3, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 minutes or 1 minute.

[0072] The present methods can be conducted on any suitable analytic instruments. In some embodiments, the present methods are conducted on a general chemistry analyzer or a clinical chemistry analyzer, e.g., general chemistry analyzer or clinical chemistry analyzer from Roche, Hitachi, Modular P, Cobas series, Beckman/Olympus AU series, Beckman Synchron and DXC series, or Abbot Architect series.

[0073] The present methods can be conducted to achieve any suitable precision. In some embodiments, the present methods can be conducted to achieve a precision or CV of about 30% or less, e.g., about 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less. For example, the present methods can be conducted to achieve a precision or CV of about 5% or less, e.g., about 5%, 4%, 3%, 2.5%, 2%, 1.5%, 1%, 0.5% or less for a vitamin D moiety level of about 30 ng/ml or less, e.g., about 30 ng/ml, 25 ng/ml, 20 ng/ml, 15 ng/ml, 10 ng/ml, 5 ng/ml, 4 ng/ml, 3 ng/ml, 2 ng/ml, 1 ng/ml, or less. In another example, the present methods can be conducted to achieve a precision or CV of about 10% or less, e.g., about 10%, 9%, 8%, 7%, 6%, 5%, 4.5%, 4.0%, 3%, 2.5%, 2%, 1.5%, 1%, 0.5% or less for a vitamin D moiety level of about 100 ng/ml or less, e.g., about 100 ng/ml, 90 ng/ml, 80 ng/ml, 70 ng/ml, 60 ng/ml, 50 ng/ml, 40 ng/ml, 30 ng/ml, 20 ng/ml, 10 ng/ml, or less.

C. Kits for Assaying a Vitamin D Moiety and Uses Thereof

[0074] In another aspect, the present invention provides a kit for assaying a vitamin D moiety in a sample, which kit comprises: a) a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH (R1); b) surface(s) or particles, e.g., latex par-

ticles, (R2) coated, e.g., separately coated, with at least two antibodies, one of the antibodies or the first antibody having a specific binding affinity towards vitamin D moiety, and another antibody or the second antibody having a specific binding affinity towards the complex formed between the first antibody and the vitamin D moiety. Optionally, the antibodies are different from a natural vitamin D binding protein for the vitamin D moiety. In some embodiments, the at least two antibodies can be attached to a same particle or same type of particle(s) or surface(s). In other embodiments, the at least two antibodies can be attached to different particles or surfaces, or different types of particles or surfaces.

[0075] Any suitable buffer of acidic pH can be used in the present kits. In some embodiments, the acidic pH buffer is the sodium acetate buffer, or sodium citrate buffer or phosphoric acid buffer.

[0076] Any suitable antibody combination comprising one antibody or the first antibody specifically binds to vitamin D moiety and the other antibody or the second antibody specifically binds to the complex formed between the first antibody and the vitamin D moiety can be used. Antibodies in any suitable forms can be used. For example, a polyclonal antibody or a monoclonal antibody, fragment of an antibody such as Fab fragment, or single chain antibody can be used. In still other embodiments, exemplary antibodies disclosed in U.S. patent publication No. 2011/0097733 A1 can be used.

[0077] The present kits can comprise any additional suitable reagents or components. In some embodiments, the present kits further comprise means for assessing binding between the specific antibodies and the vitamin D moiety to determine the presence, absence and/or amount of the vitamin D moiety in the sample.

[0078] The reagents or components in the present kits can be formulated or arranged in any suitable fashion or form. In some embodiments, the present kits comprise the following reagents: (1) a first assay reagent (R1) comprising a buffer of acidic pH; (2) a second assay reagent (R2) comprising two specific antibodies that are coated, e.g., separately coated, on surface(s) or particles, e.g., latex particles, one antibody or the first antibody having an affinity against the vitamin D moiety, and the other antibody or the second antibody having an affinity against the complex formed between the first antibody and vitamin D moiety.

[0079] The above kits can be used in a method for assaying a vitamin D moiety in a sample, which method comprises: a) forming a mixture of a sample and the first assay reagent and incubating the mixture for a period of time before adding the second assay reagent (e.g., latex particles coated with specific antibodies) to the mixture; and b) quantifying the amount of a vitamin D moiety, e.g., 25(OH)D, in the sample by measuring the optical change of the reaction mixture and using a set of vitamin D moieties, e.g., a set of 25(OH)D calibrators.

[0080] In still other embodiments, the present kits comprise (1) a first assay reagent comprising a buffer of acidic pH, and (2) a solid surface comprising an immobilized antibody having specific binding affinity towards a vitamin D moiety, and (3) a detecting reagent comprising an antibody having a binding affinity towards the complex formed between the immobilized antibody and the sample vitamin D moiety. The present kits can further comprise suitable wash-

ing reagents. The assay procedure may include wash steps between the different reagent additions.

[0081] In still another embodiment, the present kits comprise (1) a first assay reagent comprising a buffer of acidic pH; and (2) surface(s) or particles, e.g., magnetic particles, coated with an antibody having an affinity towards vitamin D moiety; and (3) an antibody labelled with a signaling molecule needed for chemiluminescence; and (4) a substrate (starter) or substrates needed for initiating the chemiluminescent reaction. The kit may also contain calibrators, controls, a dilution buffer and/or a washing buffer.

[0082] In yet another embodiment, the present invention provides a method for assaying a vitamin D moiety in a sample, which method comprises: a) contacting said sample with a first reagent comprising a buffer of acidic pH to dissociate said vitamin D moiety in said sample from its binding proteins; b) contacting said vitamin D moiety in said sample from step a) with a second reagent comprising particles, e.g., magnetic particles, coated with a first antibody that has a specific binding affinity to said vitamin D moiety to form a vitamin D moiety/first antibody complex; and c) contacting said vitamin D moiety/first antibody complex with a third reagent comprising a second antibody labelled with a signal generating moiety or molecule to form a complex among said vitamin D moiety, said first antibody and said second antibody labelled with a signal generating moiety or molecule; and d) assessing said signal from said complex among said vitamin D moiety, said first antibody complex and said second antibody to determine the presence, absence and/or amount of the vitamin D moiety in said sample. Optionally, the antibodies are different from a natural vitamin D binding protein for the vitamin D moiety. In some embodiments, the signal generating molecule is selected from the group consisting of acridinium ester, isoluminol, alkaline phosphatase, horse radish peroxidase and fluorescein.

[0083] The above kits can be used in a method for assaying a vitamin D moiety in a sample, which method comprises: a) forming a mixture of a sample and the first assay reagent and incubating the mixture for a period of time; and b) contacting the mixture with the surface(s) or particles, e.g., magnetic particles, coated with the first antibody specific for binding with vitamin D moiety, and c) after an incubation period of time, followed by a washing step, and d) re-suspending the surface(s) or particles, e.g., magnetic particles, and contacting with the second antibody which is specific for the complex formed between the first antibody on surface(s) or particles, e.g., magnetic particles, and the sample vitamin D moiety and labelled with a signaling molecule for chemiluminescence; and e) after a period of incubation followed by another washing step, the surface(s) or magnetic particles are mixed with a starter or substrates that triggers the chemiluminescent reaction. The chemiluminescent intensity (RLU) is detected to determine the presence, absence and/or amount of the vitamin D moiety in the sample.

[0084] The present reaction mixtures or kits can be formulated or arranged in any suitable fashion or manner. In some embodiments, the present reaction mixtures or kits are contained in a single phase or homogenous phase. In other embodiments, the present reaction mixtures or kits are contained in multiple phases (heterogeneous assay), e.g., two or three phases.

[0085] Optionally, the antibodies in the above kits and methods are different from a natural vitamin D binding protein for the vitamin D moiety.

[0086] The present kits can be configured to be conducted within any suitable reaction time. In some embodiments, the present kits can be configured to have a total assay time that is at about 60 minutes or shorter, e.g., about 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 minutes or 1 minute. For example, the present kits can be configured to have an assay time from initiation, e.g., addition of a sample and/or a reagent(s), to signal readout time, that is at about 60 minutes or shorter, e.g., about 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 minutes or 1 minute.

[0087] The present kits can be configured to achieve any suitable precision. In some embodiments, the present kits can be configured to achieve a precision or CV of about 30% or less, e.g., about 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less. For example, the present kits can be configured to achieve a precision or CV of about 5% or less, e.g., about 5%, 4%, 3%, 2.5%, 2%, 1.5%, 1%, 0.5% or less for a vitamin D moiety level of about 30 ng/ml or less, e.g., about 30 ng/ml, 25 ng/ml, 20 ng/ml, 15 ng/ml, 10 ng/ml, 5 ng/ml, 4 ng/ml, 3 ng/ml, 2 ng/ml, 1 ng/ml, or less. In another example, the present kits can be configured to achieve a precision or CV of about 10% or less, e.g., about 10%, 9%, 8%, 7%, 6%, 5%, 4.5%, 4.0%, 3%, 2.5%, 2%, 1.5%, 1%, 0.5% or less for a vitamin D moiety level of about 100 ng/ml or less, e.g., about 100 ng/ml, 90 ng/ml, 80 ng/ml, 70 ng/ml, 60 ng/ml, 50 ng/ml, 40 ng/ml, 30 ng/ml, 20 ng/ml, 10 ng/ml, or less.

D. Exemplary Embodiments

[0088] In some embodiments, the present invention provides a method, e.g., a homogeneous or a heterogeneous method, for determining total 25-hydroxy-vitamin D 25(OH)D concentrations in a sample, e.g., blood samples, wherein the dissociation of 25(OH)D from vitamin D binding proteins (VBP), the binding of the dissociated [25(OH)D] or free 25(OH)D with two monoclonal antibodies that forms antibody-vitamin D-antibody complex, and the detection of 25(OH)D in the sample is carried out in a single reaction mixture without phase separation or washing steps involved.

[0089] Of particular advantage of the embodiments is the significantly shorter total assay time (often <13 min in a homogenous assay format) and its user friendly homogenous assay format that eliminates the needs for sample pre-treatment or phase separation/washing steps, and that allows the assay to be easily adapted for uses on general chemistry analyzers that are routinely used in clinical laboratories.

[0090] In some embodiments, the present invention provides a method of assaying a sample of blood or blood components for determining the amount of 25-hydroxy-vitamin D comprising: a) mixing the sample with a buffer of acidic pH that is capable of dissociating 25(OH)D from its binding protein; b) adding two monoclonal antibodies that are separately coated on latex particles, with one antibody or

the first antibody having an affinity specifically towards the vitamin D moiety, and the other antibody or the second antibody having an affinity specifically towards the complex formed between the first antibody and the vitamin D moiety; and c) determining the concentration of 25(OH)D in the sample with a single reaction mixture, wherein the vitamin D binding proteins are not removed from the sample, and no phase separation or washing steps are involved.

[0091] In some embodiments, the acidic pH buffer is sodium acetate buffer containing appropriate amount of salts, polymers such as PEG 100 k and detergents. The pH of the acidic buffer can be ranged from 2.5 to 6.5. The acidic pH buffer can also be sodium citrate buffer or phosphoric acid buffer or other buffer that has a buffer function in the acidic pH range.

[0092] In some embodiments, the latex particles have the diameter sizes from 30 nm to 500 nm, preferably from 120 nm to 360 nm.

[0093] In some embodiments, the sample is biological fluids including but not limited to whole blood, plasma, serum, and urine.

[0094] In some embodiments, the concentration of 25(OH)D includes the total concentration of 25(OH)D including 25(OH)D3 and 25(OH)D2.

[0095] In some embodiments, the assay format of the homogenous method of 25(OH)D determination is the particle-enhanced immunoturbidimetric methods. In other embodiments, the assay format of the homogenous method of 25(OH)D determination is the particle-enhanced immunonephelometric methods. In still other embodiments, the assay format of 25(OH)D determination is the heterogeneous magnetic particle (beads) based immunoassay methods.

[0096] In some embodiments, the assay format of the heterogeneous method of 25(OH)D determination is an enzyme-linked immunosorbent assay (ELISA) method.

[0097] Any suitable antibodies can be used. In some embodiments, the antibody is polyclonal. In other embodiments, the antibody is monoclonal.

[0098] Any suitable particles can be used. In some embodiments, the particles are nanoparticle including but not limited to particles made of polystyrene, polymethyl methacrylate, polymethyl naphthalene, carbon 60, co-polymer of styrene, acrylic acid, naphthalene, magnetic particles, gold, silver, silica, silicon dioxide, chromium dioxide, and titanium dioxide.

[0099] In some embodiments, the present invention provides a kit for determining 25(OH)D in a sample, the kit comprises 2 reagents including (1) a buffer of acidic pH capable of dissociating 25(OH)D from its binding protein; and (2) latex particles separately coated with two monoclonal antibodies, with one antibody or the first antibody having binding affinity towards the vitamin D moiety, and the other antibody or the second antibody having a binding affinity towards the complex formed between the first antibody and the vitamin D moiety. In an exemplary assay, samples such as plasma or serum are mixed with the acidic pH buffer, and incubated for a short period of time before addition of the second reagent containing the antibodies conjugated on latex particles. The 25(OH)D concentration in the sample is quantified by measuring the optical change of the reaction mixture and using a set of 25(OH)D calibrators.

[0100] In some embodiments, the present invention provides a kit for determining 25(OH)D in a sample, the kit

comprises 2 reagents including (1) a buffer of acidic pH capable of dissociating 25(OH)D from its binding protein (partially or entirely); and (2) a reagent containing two antibodies that are separately conjugated on particles with one antibody having a binding affinity towards vitamin D moiety, and the other antibody having binding affinity towards the complex formed between the first antibody and the vitamin D moiety. In an exemplary assay, samples such as plasma or serum are mixed with the acidic pH buffer, and incubated for a short period of time before addition of the second reagent containing the antibodies coated on particles. The 25(OH)D concentration in the sample is quantified by measuring the optical change of the reaction mixture and using a set of 25(OH)D calibrators.

[0101] In some embodiments, the present invention provides a kit for determining 25(OH)D in a sample, the kit contains a dissociation solution comprising an acidic pH buffer. This kit can be used in the ELISA format with microtiter plates for 25(OH)D determination.

[0102] In some embodiments, the exemplary vitamin D assays use magnetic particles coupled with chemiluminescent or fluorescent signaling molecules. The vitamin D assay method uses magnetic particles coated with one monoclonal or polyclonal antibody having a binding affinity towards the vitamin D moiety, and uses another antibody labeled with chemiluminescent detection molecules and having a binding affinity towards the complex formed between the antibody coated on the magnetic particles and vitamin D moiety. After incubations and washing steps, the vitamin D concentrations in the samples are determined based on the signal intensity (RLU) of chemiluminescence and a calibration curve.

[0103] In some embodiments, the exemplary vitamin D assays have certain advantages over the vitamin D assays in the art. Exemplary advantages include one or more of the following: fast or fastest Vitamin D assay (results in ~10 minutes or less); universal Vitamin D assay for general chemistry analyzers; liquid stable, ready-to-use two reagents system; liquid stable calibrators and controls; automation or full automation; no requirement for pretreatments or pre-dilutions steps; equal recognition of 25-OH Vitamin D2 and 25-OH Vitamin D3; amenable to high throughput on clinical chemistry analyzers; and/or traceable to NIST SRM972. Further exemplary advantages include one or more of the following: fast testing time (less than 10 minutes); two reagent format which is a user friendly assay for vitamin D; and/or assay format adaptable to many or all automated clinical chemistry analyzers which are the most commonly used instruments in all sizes of clinical laboratories. In some embodiments, these advantages make it possible for using the exemplary vitamin D assay as a true routine clinical test.

[0104] The present invention is further illustrated by the following exemplary embodiments.

[0105] 1. A method for assaying a vitamin D moiety in a sample, which method comprises: a) contacting a sample containing or suspected of containing a vitamin D moiety with a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH, and two or more antibodies with at least one antibody or the first antibody having a binding affinity specifically towards vitamin D moiety, and another antibody (second antibody) having binding affinity towards the complex formed between the first antibody and the vitamin D moiety; and b) assessing binding between said specific antibodies and said vitamin D moiety to determine the presence, absence and/or

amount of said vitamin D moiety in said sample. The buffer of acidic pH can comprise any suitable salt, polymer and/or detergent.

[0106] 2. The method of embodiment 1, which does not comprise a step of removing the natural vitamin D binding protein for the vitamin D moiety prior to assessing binding between the specific antibodies and the vitamin D moiety.

[0107] 3. The method of embodiment 1 or 2, which does not comprise a wash step.

[0108] 4. The method of any of embodiments 1-3, which is conducted as a homogeneous assay.

[0109] 5. The method of any of embodiments 1-4, wherein the sample is contacted with a single reaction mixture comprising a buffer of acidic pH and latex particles coated with two antibodies separately, with one antibody or the first antibody having a binding affinity towards the vitamin D moiety and the other antibody or the second antibody having a binding affinity towards the complex formed between the first antibody and the vitamin D moiety.

[0110] 6. The method of any of embodiments 1-5, which does not comprise a step of contacting the sample with 8-anilino-1-naphthalenesulfonic acid ammonium salt and/or 3-(acetonylbenzyl)-4-hydroxycoumarin.

[0111] 7. The method of any of embodiments 1-5, which does not comprise a step of contacting the sample with a non-competitive displacement agent that separates the vitamin D moiety from its binding protein in the sample.

[0112] 8. The method of any of embodiments 1-7, wherein the pH in a final reaction mixture (acidic pH buffer plus sample plus latex particles) is at 3.0 or higher.

[0113] 9. The method of any of embodiments 1-8, wherein the pH in a final reaction mixture comprising the sample, the acidic pH buffer and the latex particles is at 13 or lower.

[0114] 10. The method of any of embodiments 1-9, which does not comprise a step of contacting the sample with a vitamin D releasing composition comprising a cyclodextrin, a sodium salicylate and NaOH.

[0115] 11. The method of any of embodiments 1-10, which does not comprise a step of contacting the sample with a perfluoro alkyl acid, or a salt thereof, to release 25(OH)D from vitamin D binding protein.

[0116] 12. The method of any of embodiments 1-11, which does not comprise a step of contacting the sample with a serine protease with endo- and exoproteolytic activity to digest vitamin D binding proteins in the sample.

[0117] 13. The method of any of embodiments 1-12, which is used to assess status of the vitamin D moiety in a subject, and the sample is a biological sample obtained and/or derived from the subject.

[0118] 14. The method of embodiment 13, wherein the subject is a mammal.

[0119] 15. The method of embodiment 14, wherein the mammal is a human.

[0120] 16. The method of any of embodiments 13-15, wherein the sample is a biological fluid.

[0121] 17. The method of embodiment 16, wherein the biological fluid is selected from the group consisting of whole blood, plasma, serum and urine.

[0122] 18. The method of any of embodiments 1-17, wherein the vitamin D moiety is 25-hydroxy-vitamin D₃, 25-hydroxy-vitamin D₂, a vitamin D metabolite or 1,25-dihydroxy-vitamin D₃ (1,25-(OH)₂D₃).

[0123] 19. The method of embodiment 18, wherein the vitamin D metabolite is 25-hydroxy-vitamin D (25(OH)D).

[0124] 20. The method of embodiment 19, wherein the 25(OH)D is 25(OH)D3.

[0125] 21. The method of embodiment 19, wherein the 25(OH)D is 25(OH)D2.

[0126] 22. The method of embodiment 19, wherein the 25(OH)D is a sum of 25(OH)D2 and 25(OH)D3.

[0127] 23. The method of any of embodiments 1-22, wherein the acidic pH buffer is sodium acetate buffer containing suitable amount of salts such as NaCl, polymers such as PEG 100 k and detergent such as Tween 20. The acidic pH buffer can also be sodium citrate buffer, or phosphoric acid buffer, or a combination thereof.

[0128] 24. The method of embodiment 23, wherein the pH of the acidic buffer is ranging from 2.5 to 6.5, preferably from 4.0 to 5.5.

[0129] 25. The method of any of embodiments 1-24, wherein the monoclonal antibodies are those with at least one antibody or the first antibody having a specific binding affinity against the vitamin D moiety and with another antibody or the second antibody having a specific binding affinity against the complex formed between the first antibody and the vitamin D moiety.

[0130] 26. The method of embodiment 25, wherein one antibody or the first antibody specifically binds to 25(OH)D3 or 25(OH)D2.

[0131] 27. The method of embodiment 25, wherein one antibody or the second antibody specifically binds to the complex formed between the first antibody and the vitamin D moiety.

[0132] 28. The method of embodiments 25-27, wherein the antibodies are monoclonal antibodies.

[0133] 29. The method of embodiment 25-27, wherein the antibodies are polyclonal antibodies or combination of monoclonal antibody and polyclonal antibodies.

[0134] 30. The method of any of embodiments 1-29, which is conducted using a particle-enhanced immunoturbidimetric method.

[0135] 31. The method of any of embodiments 1-29, which is conducted using a particle-enhanced immunonephelometric method.

[0136] 32. The method of any of embodiments 1-29, which is conducted using a magnetic particle (beads) based immunoassay method.

[0137] 33. The method of any of embodiments 30-33, wherein the particle comprises polystyrene, polymethyl methacrylate, polymethyl naphthalene, poly(divinylbenzene), polyvinyl naphthalene, co-polymer of styrene, acrylic acid divinylbenzene, naphthalene, carbon 60, magnetic beads, gold, silver, silica, silicon dioxide, chromium dioxide, and/or titanium dioxide.

[0138] 34. The method of any of embodiments 30-33, wherein the particle is a nanoparticle.

[0139] 35. The method of embodiment 34, wherein the nanoparticle has a diameter ranging from about 30 nm to about 500 nm.

[0140] 36. The method of any of embodiments 1-35, which is conducted using a homogeneous or a heterogeneous assay format.

[0141] 37. The method of any of embodiments 1-36, which is conducted using a sandwich or competitive assay format.

[0142] 38. The method of any of embodiments 1-37, which is a homogenous assay conducted in a single reaction mixture without phase separation or washing step.

[0143] 39. The method of any of embodiments 1-38, the total assay time for homogeneous format is less than 30 min, typically less than 15 min, and the total assay time for heterogeneous assay format is less than 60 min, typically less than 45 min.

[0144] 40. The method of any of embodiments 1-39, which is conducted on a general chemistry analyzer or a clinical chemistry analyzer.

[0145] 41. The method of embodiment 40, wherein the general chemistry analyzer or clinical chemistry analyzer includes but not limited to Roche, Hitachi, Modular P, Cobas series, Beckman/Olympus AU series, Beckman Synchron and DXC series, or Abbot Architect series.

[0146] 42. A kit for assaying a vitamin D moiety in a sample, which kit comprises: a) a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH; and b) particles, e.g., latex particles, coated, e.g., separately coated, with at least two antibodies of which one, or the first one, has a specific binding affinity towards the vitamin D moiety, and another antibody, or the second antibody, has a specific binding affinity towards the complex formed between the first antibody and the vitamin d moiety.

[0147] 43. The kit of embodiment 42, which further comprises means for assessing binding between vitamin D moiety and the antibodies coated on latex particles to determine the presence, absence and/or amount of the vitamin D moiety in the sample.

[0148] 44. The kit of embodiment 43, which comprises reagents: (1) a first assay reagent comprising a buffer of acidic pH containing salts, polymer and detergent; (2) a second assay reagent comprising a suspension of latex particles coated with at least two antibodies with one antibody or the first antibody having a binding affinity towards the vitamin D moiety, and the other antibody or the second antibody or antibodies having a specific binding affinity towards the complex formed between the first antibody and the vitamin D moiety.

[0149] 45. The kit of embodiment 44, which comprises: (1) a first assay reagent comprising a buffer of acidic pH; and (2) microtiter plate wells containing an immobilized antibody that specifically binds to a vitamin D moiety or its analog.

[0150] 46. A method for assaying a vitamin D moiety in a sample using the kit of embodiment 44, which method comprises: a) forming a mixture of a sample, the first assay reagent for a period of time before adding the second assay reagent to the mixture; and b) quantifying the amount of 25(OH)D in the sample by measuring the optical change of the reaction mixture and using a set of 25(OH)D calibrators.

[0151] 47. A method for assaying a vitamin D moiety in a sample using the kit of embodiment 45, which method comprises: a) forming a mixture of a sample and the first assay reagent in the microtiter plate wells, and incubating the mixture for a period of time; and b) contacting the mixture with the second antibody labelled with peroxidase (HRP) and incubating for a period of time; c) after a washing step, HRP substrate is added to the microtiter plate well and the color is developed for quantifying the presence, absence and/or amount of the vitamin D moiety in the sample.

[0152] 48. A method for assaying a vitamin D moiety in a sample using the kit comprises of reagents including a buffer of acidic pH; magnetic particles coated with an antibody or the first antibody that specifically binds vitamin D moiety,

and the second antibody that is labelled with signaling molecules and having a specific binding affinity towards the complex formed between the first antibody coated on the magnetic particles and the vitamin D moiety, wherein the signaling molecules are acridinium ester, isoluminol, alkaline phosphatase, horse radish peroxidase, fluororesin or any other chemiluminescence or fluorescence based signal molecules commonly used for signal detections.

[0153] 49. A method for assaying a vitamin D moiety in a sample using the kit of embodiment 48, which method comprises: a) forming a mixture of a sample, the acidic pH buffer and magnetic particles coated with the first antibody, and incubating for a period of time; b) washing the magnetic particles and adding the second antibody labelled with signaling molecules and having a specifically binding affinity to the complex formed between the first antibody coated on the magnetic particles and the sample vitamin D moiety followed by a period of incubation time; c) washing the magnetic particles and adding the substrates (starters) if needed for the signal detection by the method of chemiluminescent or fluorescent detection.

[0154] 50. A method for assaying a vitamin D moiety in a sample using the kit of embodiment 49, which method comprises: a) forming a mixture of a sample, the acidic pH buffer, the first antibody coated on magnetic particles and the second antibody labeled with signaling molecules and has a specific binding affinity towards the complex formed between the first antibody coated on the magnetic particles and the sample vitamin D moiety, and incubating for a period of time; b) washing the magnetic particles and addition of the substrates if needed for the signal detection by the method of chemiluminescent or fluorescent method.

[0155] 51. The method of any of embodiments 1-41, wherein the another antibody (second antibody) specifically binds to an epitope on the vitamin D moiety that is not bound by one of the antibodies (or the first antibody).

[0156] 52. The method of any of embodiments 1-41, wherein the another antibody (second antibody) binds or specifically binds to the one of the antibodies (or the first antibody).

EXAMPLES

Example 1: 25(OH)D Assay Kit

[0157] The latex particle enhanced immunoassay reagents used in this example are listed below:

Reagent 1

0.05M Sodium Acetate, pH 4.0

10% NaCl

5% Choline Chloride 0.5M MES

[0158] 0.5% sugar

0.04% Tween 20

0.9% PEG 100 k

0.09% NaN₃

Reagent 2

0.6 M Tris, pH 8.0

0.1% BSA

10% Sucrose

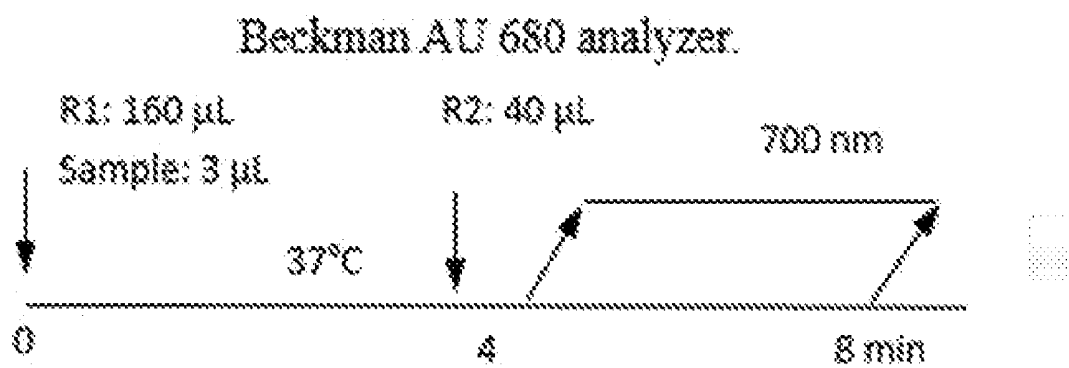
0.2% Tween 20

[0159] 0.05% first antibody-latex particle conjugate

0.08% second antibody-latex particle conjugate

0.09% NaN₃

[0160] 1. Assay Procedure with Beckman AU 680 Analyzer



[0161] Three (3) μL of sample was mixed with 160 μL of Reagent 1 inside a cuvette and incubated at 37° C. for about 3.5 min. Forty (40) μL of Reagent 2 was added to the mixture and incubated for about 4 min. The change in absorbance at 700 nm was measured using the assay parameters as indicated in the parameter sheet (Table 1) below.

TABLE 1

Assay Parameters on AU 680 analyzer			
General			
Test Name: VITD	Type: Serum	Operation: Yes	
Sample Volume 3.0 µl	Dilution 0 µl	Pre-Dilution Rate 1	
Reagents:		Min OD	Max OD
R1 volume 160 µl Dilution 0 µl		L:	H:
R2 volume 40 µl Dilution 0 µl			
Wavelength:		Reagent OD Limit:	
Method: FIXED		First L: -2.000; First H: 3.000	
Reaction Slope: +		Last L: -2.000; Last H: 3.000	
Measuring Point 1: First 11; Last 22		Dynamic Range:	
Measuring Point 2: First; Last		L: H:	
Linearity:		Correlation Factor:	
No-Lag-Time: No		A: 1.0000 B: 0.000	
		Onboard stability Period	
Calibration Type: 5AB Formula: Spline Counts: 2 Process: CONC			
Cal No.	CONC	Factor/OD-L	Factor OD-HOD
Point 1:1	0.00	-2.00000	3.0000
Point 2:2	*. **	-2.00000	3.0000
Point 3:3	*. **	-2.00000	3.0000
Point 4:4	*. **	-2.00000	3.0000
Point 5:5	*. **	-2.00000	3.0000
Point 6:6			
Point 7:			
1-Point Cal. Point	__with CONC-0	Advanced Calibration: No	
MB type Factor:		Calibration Stability Period:	

[0162] 1. Assay Calibration Curve (Example Only)

[0163] The 25(OH)D Assay kit was calibrated with serum based calibrators of known 25(OH)D concentrations. The calibration curve is shown in FIG. 1.

[0164] After successful calibration of the 25(OH)D assay kit, controls and linearity standards were tested. In addition, patient serum samples were tested and the results were compared with a FDA approved, commercially available vitamin D assay method (DiaSorin immunoassay method) as well as with a liquid chromatography-mass spectrophotometric (LC-MS) method for total vitamin D (considered as the gold standard for vitamin D test). The results are shown in Tables 1-3 below and FIGS. 2-6.

[0165] 2. Results:

[0166] Assay precisions are illustrated in Table 2 below.

TABLE 2

Vitamin D assay precisions Precision							
	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
Rep. 1	5.0	12.1	24.5	46.1	59.3	101.2	157.1
Rep. 2	6.6	12.5	25.3	46.5	59.9	100.2	158.9
Rep. 3	5.9	12.5	24.5	46.0	60.7	102.1	154.8
Rep. 4	5.7	12.5	23.9	46.8	59.3	100.6	158.2
Rep. 5	3.5	11.8	24.3	46.8	60.4	103.4	155.7
Rep. 6	4.7	12.0	24.8	46.9	60.0	99.2	157.3
Rep. 7	5.7	12.0	24.4	46.1	60.0	102.0	163.4

TABLE 2-continued

Vitamin D assay precisions Precision							
	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	Level 7
Rep. 8	5.2	12.5	24.8	46.4	59.7	101.5	158.4
Rep. 9	4.2	12.5	24.7	47.1	59.9	102.6	161.6
Rep. 10	4.9	12.1	24.6	46.9	60.2	100.8	159.1
Rep. 11	3.5	12.5	24.6	47.1	60.5	103.3	161.1
Rep. 12	2.9	12.3	24.9	46.4	60.4	101.7	155.7
Mean (ng/ml)	4.8	12.3	24.6	46.6	60.0	101.6	158.4
% CV	23.1%	2.1%	1.4%	0.8%	0.7%	1.2%	1.6%

[0167] Assay linearity on AU 680 analyzer is illustrated in FIG. 2. Assay accuracy in comparison with DiaSorin Liason method is illustrated in FIG. 3. Assay accuracy in comparison with LC-MS/MS method is illustrated in FIG. 4.

Example 2: 25(OH)D Assay Kit

[0168] Magnetic particle based chemiluminescent immunoassay reagents used are listed in this example:

[0169] Reagent 1:

[0170] 0.05M Sodium Acetate buffer, pH 4.0

[0171] 8% NaCl

[0172] 1.2% Sucrose

[0173] 0.05% Tween 20

[0174] 0.09% NaN₃

[0175] Reagent 2:**[0176]** 1×PBS buffer, pH 7.4**[0177]** 0.3% BSA**[0178]** 0.04% Tween 20**[0179]** 0.09% NaN₃**[0180]** 0.1 mg/ml magnetic particles coated with the first antibody**[0181]** Reagent 3:**[0182]** 1×PBS buffer, pH 8.0**[0183]** 0.2% BSA**[0184]** 0.04% Tween 20**[0185]** 1 ug/ml ABEI-labelled second antibody**[0186]** NaN₃ 0.09%**[0187]** Starter (Substrates):**[0188]** a) 5% NaOH; b) 0.1% Peroxide**[0189]** Washing Solution:**[0190]** 0.1 M Tris-HCl buffer and detergent.**[0191]** Assay Procedure Using a Commercial Available Instrument:

[0192] Five ul (5 ul) of serum Sample was mixed with 180 ul of the Reagent 1, and incubate for 5 min to dissociate the vitamin D in the sample from its binding proteins. Aspirate 100 ul of the above sample/reagent 1 mixture, and mix with 20 ul of the Reagent 2, and incubate for 20 min before addition of 50 ul of Reagent 3. After another 10 min of incubation, the magnetic particles were washed by the washing buffer for 3 times. 200 ul of Substrates were added and the chemiluminescent intensity or RLU was read for 3 seconds. The vitamin D content in the sample was determined based on the RLU and the calibration curve established under the same assay conditions.

[0193] Results:

[0194] An exemplary calibration curve of chemiluminescent detection method is illustrated in FIG. 5. Assay precisions (10 repeats for each level of controls) are illustrated in Table 3 below. Comparative data between an exemplary method of the present disclosure and a commercially available predicate method (immunoassay) on assay accuracy is illustrated in FIG. 6.

TABLE 3

Assay precisions (10 repeats for each level of controls)			
	Vit. D Control I	Vit. D Control II	Vit. D Control III
Conc. ng/ml	10.5	30.1	60.9
CV %	3.4%	2.9%	2.2%

Example 3: Analytical Performance

a. Precision/Reproducibility

[0195] Precision of an exemplary Diazyme vitamin D assay (latex particle enhanced immunoassay in Example 1) was evaluated according to the Clinical and Laboratory Standards Institute (CLSI) EP5-A2 guideline. Precision assessment was performed on the Beckman AU680 chemistry analyzer. In the study, three lots of reagents were used. For each reagent lot, 12 specimens were tested: 2 vitamin D controls and 10 vitamin D human serum samples. The tested serum samples coming with IRB-approval were obtained from a commercial source (PromedDx) and covered the dynamic range of the assay. As recommended by the CLSI EP5-A2 protocol, precision samples were tested in dupli-

cates per run and two runs per day over 20 working days. Eighty (80) data points were obtained per specimen and per reagent lot.

[0196] The precision results obtained for one representative lot of reagents is summarized Table 4 below.

TABLE 4

Sample	Mean	Within-Run		Total	
	(N = 80)	SD	% CV	SD	% CV
Control 1	22.3	0.92	4.2%	1.33	6.0%
Control 2	43.7	1.07	2.4%	1.40	3.2%
Sample 1	11.1	0.87	7.8%	1.86	16.7%
Sample 2	14.1	0.75	5.3%	2.14	15.1%
Sample 3	18.6	0.86	4.6%	1.69	9.0%
Sample 4	22.1	0.86	3.9%	1.41	6.4%
Sample 5	43.4	0.89	2.1%	1.22	2.8%
Sample 6	59.7	1.17	2.0%	1.89	3.2%
Sample 7	80.6	1.32	1.6%	2.31	2.9%
Sample 8	99.8	2.32	2.3%	3.25	3.3%
Sample 9	118.5	2.27	1.9%	4.11	3.5%
Sample 10	140.0	3.58	2.6%	4.34	3.1

b. Linearity/Assay Reportable Range

[0197] Linearity of the exemplary Diazyme vitamin D assay was evaluated using the CLSI EP6-A guideline (Evaluation of the Linearity of Quantitative Analytical Methods; approved: 2003).

[0198] Acceptance Criteria

[0199] Linear regression of measured values versus expected values has a slope of 1.0 ± 0.05 and a correlation coefficient $R^2 > 0.95$.

[0200] The linearity study was performed on the Beckman AU680 chemistry analyser. A human serum sample was spiked with a vitamin D stock solution to a concentration of 156.3 ng/mL (as measured in triplicates using the exemplary Diazyme vitamin D assay). Vitamin D stock material was purchased from Sigma-Aldrich and molar concentrations were calculated using the information provided by the certificate of analysis. Thus prepared high sample was diluted with a low sample measuring 4.3 ng/mL (as measured in triplicates using the exemplary Diazyme vitamin D assay) to create a total of 11 linearity levels as follows:

Level 01: 1.00 ml Low Sample+0.00 ml of High Sample

Level 02: 0.90 ml Low Sample+0.10 ml of High Sample

Level 03: 0.80 ml Low Sample+0.20 ml of High Sample

Level 04: 0.70 ml Low Sample+0.30 ml of High Sample

Level 05: 0.60 ml Low Sample+0.40 ml of High Sample

Level 06: 0.50 ml Low Sample+0.50 ml of High Sample

Level 07: 0.40 ml Low Sample+0.60 ml of High Sample

Level 08: 0.30 ml Low Sample+0.70 ml of High Sample

Level 09: 0.20 ml Low Sample+0.80 ml of High Sample

Level 10: 0.10 ml Low Sample+0.90 ml of High Sample

Level 11: 0.00 ml Low Sample+1.00 ml of High Sample

[0201] The linearity set prepared above was tested with the exemplary Diazyme vitamin D assay in triplicate using one lot of reagents. The linearity test results are shown in the Table 5 below and in FIG. 7.

TABLE 5

Level	Rep. 1	Rep. 2	Rep. 3	Measured (ng/mL)	Expected (ng/mL)	% Deviation
Level 1	3.5	4.4	5.0	4.3	4.3	0.0%
Level 2	22.4	21.3	20.4	21.4	19.5	9.6%
Level 3	38.0	35.6	37.6	37.1	34.7	6.8%
Level 4	49.7	53.7	51.0	51.5	49.9	3.1%
Level 5	66.4	66.1	65.4	66.0	65.1	1.3%
Level 6	83.6	78.9	81.4	81.3	80.3	1.2%
Level 7	94.0	94.1	91.9	93.3	95.5	-2.3%
Level 8	113.0	110.7	110.0	111.2	110.7	0.5%
Level 9	126.9	122.4	121.1	123.5	125.9	-2.0%
Level 10	143.7	138.0	138.9	140.2	141.1	-0.7%
Level 11	157.3	157.1	154.6	156.3	156.3	0.0%

Example 4: Comparison Studies

a. Method Comparison with Predicate Device

[0202] For this method comparison study, individual serum samples were tested with the exemplary Diazyme vitamin D assay (latex particle enhanced immunoassay in Example 1) and compared to the predicate device (DiaSorin Liaison LX). The serum samples were obtained from a commercial source, Biochemed and came with IRB-approval. Samples that returned values outside the dynamic range of the assay were excluded. A total of 40 unaltered samples spanning assay AMR were used in the assessment of accuracy. Results are shown FIG. 8.

b. Matrix Comparison

[0203] To evaluate the effect of anticoagulants, the exemplary Diazyme vitamin D assay was used to measure the 25-OH Vitamin D concentrations of matched sets of serum, K₂-EDTA, K₃-EDTA plasma and Li-Heparin plasma. The samples used in this study were obtained from a certified commercial source (ProMedDx, LLC). All tested samples were collected according to an IRB approved protocol by the commercial vendor. The reported values for each sample and for each matrix were obtained from single measurements. The total number of matched sets tested was 54. In order to cover the claimed measuring range for each matrix, seven spiked patient samples were included in the study. Results are shown FIGS. 9-11.

Example 5: Exemplary Diazyme Vitamin D Chemiluminescence Assay

[0204] An exemplary Diazyme vitamin D chemiluminescence assay is composed of 3 reagents: magnetic beads conjugated with the first antibody; extraction buffer; and the second antibody conjugated with ABEI molecule (ISO-lumina) as described in Example 2. The assay procedure is shown in Table 6 below:

TABLE 6

Assay procedure Diazyme Chemiluminescence Assay Format	
Sample/acidic buffer/Beads	2 ul + 50 ul + 20 ul
Incubation	15 min
ABEI/AE Label	150 ul
Incubation	15 min
Cycle washing (3 cycles)	400 ul
Measurement	3 seconds

[0205] The exemplary Diazyme vitamin D chemiluminescence Assay was compared to Roche VD assay. The comparison results are shown in FIG. 12. Precision of the exemplary Diazyme vitamin D chemiluminescence Assay is shown in Tables 7 and 8 below.

TABLE 7

Assay precisions	
Sample	Vitamin D (ng/ml)
1	17.5
2	16.3
3	17.4
4	17.6
5	16.8
6	16.9
7	16.9
8	17.3
9	16.5
10	16.5
11	16.9
12	17.4
13	16.4
14	16.1
15	16.6
16	16.3
17	16.9
18	17.1
19	17.2
20	17.2
Mean	16.9
Stdev	0.4
CV	2.6%

TABLE 8

Assay precisions	
Sample	Vitamin D (ng/ml)
21	70
22	64.2
23	68.6
24	64.5
25	60.0
26	69.8
27	63.1
28	66.7
29	67.2
30	65.1
31	67.4
32	65.5
33	64.7
34	67.4
35	69.6
36	60.9
37	64.5
38	66.4
39	60.4
40	63.9
Mean	65.5
Stdev	3.0
CV	4.5%

[0206] The above examples are included for illustrative purposes only and are not intended to limit the scope of the invention. Many variations to those described above are possible. Since modifications and variations to the examples described above will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

1. A method for assaying a vitamin D moiety in a sample, which method comprises:

- a) contacting a sample containing or suspected of containing a vitamin D moiety with a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH, and at least two antibodies, e.g., at least two monoclonal antibodies, that are attached to, e.g., separately conjugated to, surface(s) or particles, e.g., nanoparticles, wherein at least one of said antibodies (or the first antibody) has a specific binding affinity towards the vitamin D moiety, and at least another said antibody (or the second antibody) has a specific binding affinity towards the complex formed between the first antibody and the vitamin D moiety, and optionally said antibodies are different from a natural vitamin D binding protein for said vitamin D moiety; and
- b) assessing binding between said antibodies, e.g., said specific antibodies, and said vitamin D moiety to determine the presence, absence and/or amount of said vitamin D moiety in said sample.

2. The method of claim 1, which does not comprise a step of removing a natural vitamin D binding protein from the sample prior to assessing binding between the antibodies and the vitamin D moiety, and/or does not comprise a wash step.

3. The method of claim 1, which is conducted as a homogeneous assay.

4. The method of claim 1, wherein the sample is contacted with a single reaction mixture comprising a buffer of acidic pH capable of dissociating the vitamin D moiety from its binding proteins, and paired antibodies separately conjugated onto particles, e.g., nanoparticles, with one antibody or the first antibody having a specific binding affinity towards the vitamin D moiety and the other antibody or the second antibody having a specific binding affinity towards the complex formed between the first antibody and the vitamin D moiety.

5. The method of claim 1, wherein the pH in a final reaction mixture comprising the sample, the acidic pH buffer and antibodies conjugated on particles, e.g., nanoparticles, ranges from about 3.0 to about 13, preferably from about 5.5 to about 8.5.

6. The method of claim 1, which is used to assess status of the vitamin D moiety in a subject, and the sample is a biological sample obtained and/or derived from the subject.

7. The method of claim 1, wherein the vitamin D moiety is 25(OH)D₂, 25(OH)D₃ or a sum of 25(OH)D₂ and 25(OH)D₃.

8. The method of claim 1, wherein the acidic pH buffer is the sodium acetate buffer, or sodium citrate buffer, or phosphoric acid buffer, a combination thereof, or any buffer having a buffer function in the acidic pH range.

9. The method of claim 8, wherein the pH of the acidic buffer is in the range from about 2.5 to about 6.9.

10. The method of claim 1, wherein the antibodies are paired monoclonal antibodies with one antibody or the first antibody having a specific binding affinity towards the vitamin D moiety, and the other antibody or the second antibody having a specific binding affinity towards the complex formed between the first antibody and the vitamin D moiety.

11. The method of claim 1, wherein the antibodies are polyclonal antibodies or combination of monoclonal antibody and polyclonal antibodies.

12. The method of claim 10, wherein the first antibody specifically binds to 25(OH)D, and the second antibody specifically binds to the complex formed between the first antibody and 25(OH)D.

13. The method of claim 10, wherein the first antibody and the second antibody are separately conjugated to particles, e.g., nanoparticles, and used either as a single combined reagent or used as two separated reagents in the assay procedure.

14. The method of claim 1, wherein the acidic pH buffer contains a salt, polymer, and/or a detergent that allows for partial or total dissociation of vitamin D moiety from its binding protein(s) within a short period of time (e.g., less than 30 min).

15. The method of claim 14, wherein the salt, polymer, and/or a detergent is included in the acidic pH buffer, or in a separated reagent solution used to mix with the acidic pH buffer in the assay procedure.

16. The method of claim 1, wherein the particles, e.g., nanoparticles, comprise polystyrene, polymethyl methacrylate, polymethyl naphthalene, poly(divinylbenzene), polyvinyl naphthalene, co-polymer of styrene, acrylic acid divinylbenzene, naphthalene, carbon 60, magnetic beads, gold, silver, silica, silicon dioxide, chromium dioxide, and/or titanium dioxide.

17. The method of claim 1, which is conducted using a particle-enhanced immunoturbidimetric method, a particle-enhanced immunonephelometric method or a magnetic particle (bead) based immunoassay method.

18. The method of claim 17, wherein latex particles are used, and the particle sizes (diameters) range from about 30 nm to about 500 nm.

19. The method of claim 17, when magnetic particles are used, the particle sizes (diameters) can range from about 500 nm to about 2,000 nm, optionally the first antibody is coated on the magnetic particles and the second antibody is labeled with chemiluminescent or fluorescent signal generating molecules such as acridinium ester, isoluminol, alkaline phosphatase and horse radish peroxidase, or fluororesin or green proteins, and further optionally the assay is conducted in a heterogeneous format involving one or two washing steps.

20. The method of claim 19, wherein the assay is a heterogeneous assay involving phase separation step or steps.

21. The method of claim 1, wherein the assay is completed within 30 min or less.

22. The method of claim 1, wherein the assay is conducted with a general chemistry analyzer or a clinical chemistry analyzer.

23. The method of claim 1, wherein the assay is conducted in a homogeneous format without a need to physically remove any substrates such as vitamin D binding proteins from the samples in or before or during the assay process.

24. The method of claim 19, wherein the assay is completed within 60 min or less.

25. A kit for assaying a vitamin D moiety in a sample, which kit comprises:

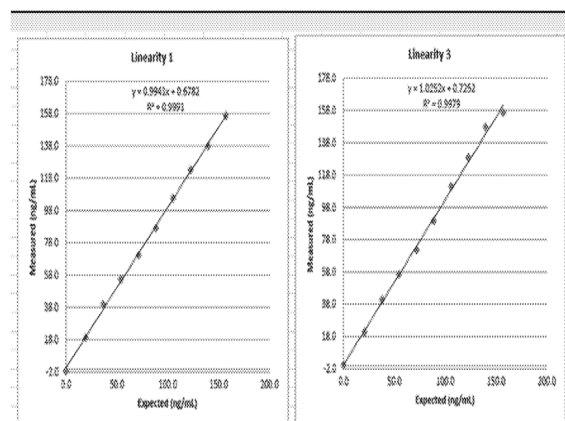
- a) a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH;

- b) at least two antibodies, e.g., at least two monoclonal antibodies, that are attached to, e.g., separately conjugated to, surface(s) or particles, e.g., latex particles, wherein at least one of said antibodies (or the first antibody) has a specific binding affinity towards the vitamin D moiety, and at least another said antibody (or the second antibody) has a specific binding affinity towards the complex formed between the first antibody and the vitamin D moiety, if present in said sample, and optionally said antibodies are different from a natural vitamin D binding protein for said vitamin D moiety.
- 26.** The kit of claim **25**, which further comprises means for assessing binding between the at least two antibodies and the vitamin D moiety to determine the presence, absence and/or amount of the vitamin D moiety in the sample.
- 27.** The kit of claim **25**, which comprises:
- a first assay reagent comprising a buffer of acidic pH containing a salt, a polymer and/or a detergent, and
 - a second assay reagent comprising a paired antibodies separately conjugated on particles, e.g., nanoparticles, with one antibody or the first antibody having a specific binding affinity to 25(OH)D, and with another antibody or the second antibody having a specific binding affinity to the complex formed between the first antibody and the 25(OH)D.
- 28.** A method for assaying a vitamin D moiety in a sample using the kit of claim **25**, which method comprises:
- forming a mixture of a sample and the first assay reagent and incubating the mixture for a period of time before adding the second assay reagent to the mixture; and
 - quantifying the amount of 25(OH)D in the sample by measuring the optical change of the reaction mixture and using a set of 25(OH)D calibrators.
- 29.** A method for assaying a vitamin D moiety in a sample using the kit of claim **25**, which method comprises:
- contacting the sample with the first antibody that is coated on a microtiter plate in the presence of the first assay reagent, and incubating the mixture for a period of time; and
 - adding the second antibody that is labelled with a signaling molecule (e.g., HRP), and
 - after incubation and washing step(s), determining the presence, absence and/or amount of the vitamin D moiety in the sample based on the signal intensity and the calibration curve.
- 30.** A kit for assaying a vitamin D moiety in a sample, which kit comprises:
- a buffer of acidic pH;
 - particles, e.g., magnetic particles, coated with a first antibody that has a specific binding affinity to said vitamin D moiety;
 - a second antibody labelled with a signal generating molecule selected from acridinium ester, isoluminol, alkaline phosphatase, horse radish peroxidase or fluorescein.
 - substrate(s) solution and/or starter needed for chemiluminescent or fluorescent signal generation.
- 31.** The kit of claim **30**, which further comprises the substrate(s) solution and/or starter.
- 32.** The kit of claim **30**, which is configured for a heterogeneous assay involving phase separation step(s) and/or washing step(s) before signal detection.
- 33.** An article of manufacture for assaying a vitamin D moiety in a sample, which article of manufacture comprises a kit of claim **30**, and substrate(s) solution and/or starter needed for chemiluminescent or fluorescent signal generation.
- 34.** The method of claim **1**, wherein the another antibody (second antibody) specifically binds to an epitope on the vitamin D moiety that is not bound by one of the antibodies (or the first antibody).
- 35.** The method of claim **1**, wherein the another antibody (second antibody) binds or specifically binds to the one of the antibodies (or the first antibody).
- 36.** A method for assaying a vitamin D moiety in a sample, which method comprises:
- contacting said sample with a first reagent comprising a buffer of acidic pH to dissociate said vitamin D moiety in said sample from its binding proteins;
 - contacting said vitamin D moiety in said sample from step a) with a second reagent comprising particles, e.g., magnetic particles, coated with a first antibody that has a specific binding affinity to said vitamin D moiety to form a vitamin D moiety/first antibody complex; and
 - contacting said vitamin D moiety/first antibody complex with a third reagent comprising a second antibody labelled with a signal generating moiety or molecule to form a complex among said vitamin D moiety, said first antibody and said second antibody labelled with a signal generating moiety or molecule; and
 - assessing said signal from said complex among said vitamin D moiety, said first antibody complex and said second antibody to determine the presence, absence and/or amount of the vitamin D moiety in said sample.
- 37.** The method of claim **36**, wherein the signal generating moiety or molecule is selected from the group consisting of acridinium ester, isoluminol, alkaline phosphatase, horse radish peroxidase and fluorescein.
- 38.** A method for assaying a vitamin D moiety in a sample, which method comprises:
- contacting a sample containing or suspected of containing a vitamin D moiety with a buffer that is capable of dissociating a vitamin D moiety from its binding protein and/or a buffer of acidic pH, and at least two antibodies, e.g., at least two monoclonal antibodies, wherein at least one of said antibodies (or the first antibody) has a specific binding affinity towards the vitamin D moiety and is attached to a solid surface, e.g., solid surface of a microtiter plate or a particle, and at least another said antibody (or the second antibody) has a specific binding affinity towards the complex formed between said first antibody and said vitamin D moiety and is labelled with a signal generating moiety, and
 - assessing binding between said antibodies, e.g., said specific antibodies, and said vitamin D moiety to determine the presence, absence and/or amount of said vitamin D moiety in said sample.
- 39.** The method of claim **38**, wherein the antibodies are different from a natural vitamin D binding protein for the vitamin D moiety.
- * * * * *

专利名称(译)	用于测定维生素D部分的方法和试剂盒		
公开(公告)号	US20180031581A1	公开(公告)日	2018-02-01
申请号	US15/663578	申请日	2017-07-28
[标]发明人	YUAN CHONG SHENG SAIDA FAKHRI BEN HABIB		
发明人	YUAN, CHONG-SHENG SAIDA, FAKHRI BEN HABIB		
IPC分类号	G01N33/82 G01N33/53 A61K31/593		
CPC分类号	G01N33/82 A61K31/593 G01N33/53		
优先权	62/368940 2016-07-29 US		
外部链接	Espacenet USPTO		

摘要(译)

本发明提供用于测定样品中维生素D部分的方法和组合物，例如试剂盒，其包含或使用能够将维生素D部分与其结合蛋白和/或其缓冲液解离的缓冲液。酸性pH和至少两种抗体，例如至少两种单克隆抗体，其分别与颗粒缀合，例如乳胶颗粒，其中至少一种所述抗体（或第一抗体）对维生素具有特异性结合亲和力如果存在于所述样品中，D部分和至少另一种所述抗体（或第二抗体）对第一抗体和维生素D部分之间形成的复合物具有特异性结合亲和力。在一些实施方案中，测量由于抗体和维生素D部分之间的凝集反应引起的光学变化，以确定样品中维生素D的含量。还提供了用于测定样品中维生素D部分的试剂盒和反应混合物。



Assay linearity on AU 680 analyzer