SPECIAL GUEST EDITOR SECTION

A Direct Assay for Measuring Free 25-Hydroxyvitamin D

NICOLAS HEUREUX
DIAsource ImmunoAssays, 2 rue du Bosquet, 1348 Louvain-la-Neuve, Belgium
ERNST LINDHOUT and LEON SWINKELS
Future Diagnostics Solutions, 279 Nieuweweg, 6603 BN Wijchen, The Netherlands

Recent studies suggest that the concentration and genotype of vitamin D binding protein (VDBP) are important factors that determine the bioavailability of 25-hydroxyvitamin D [25(OH)D] in blood. Accumulating data indicate that, e.g., in pregnant women, hemodialysis patients, chronic kidney disease, liver failure, and bladder and pancreatic cancers, the measurement of free 25(OH)D in serum provides more relevant diagnostic information than measurement of total 25(OH)D. The aim of this study was to develop and validate an ELISA for direct measurement of free 25(OH)D in serum. A simple and direct ELISA was developed, based on a two-step immunoassay procedure performed in a microtiter plate. The assay has been characterized in terms of precision (4–10% CV, according to concentration), sensitivity (limits of blank = 0.5–1.0 pg/mL and LODs = 1.3–1.8 pg/mL), accuracy (correlation to dialysis, ELISA = 0.99xdialysis-0.5 pg/mL, r^2 = 0.74), cross-reactivity of the antibody for the D_2 form (77%), and addition of both VDBP and albumin (35–38% recovery upon addition of VDBP, 53–58% upon addition of albumin). The assay has already been used in multiple studies, including its comparison with calculation methods and in studies of patients with liver failure, different ethnic groups, supplemented mice, respiratory diseases, and obesity. The free 25(OH)D ELISA can be used in studies as a valuable tool to establish the clinical relevance of free 25(OH)D.

Due to its hydrophobic nature, 25(OH)D circulates on binding proteins. About 90% of the total circulating 25(OH)D is bound to the vitamin D binding protein (VDBP), whereas the remaining 10% is bound to albumin (1). Only a tiny fraction, about 0.04%, circulates in the free form. Until recently, little attention has been paid to the free fraction of 25(OH)D, and no simple, direct method was available for its measurement.

Nowadays, nobody questions the measurement of free thyroid hormones such as T3 and T4, which have almost completely replaced the measurement of total T3 and T4 in clinical laboratories worldwide (2). Like vitamin D, the metabolites T3 and T4 are small hydrophobic molecules and circulate on binding proteins in the aqueous environment of the human body. However, according to the free hormone hypothesis, the small fraction of T3 and T4 that circulates unbound as the free form represents the biologically active portion of the hormones and is thought by the scientific community to be a more accurate reflection of thyroid hormone function than the total concentrations of these molecules (3). This concept is not confined to thyroid hormones; e.g., the measurement of free testosterone is now also part of the laboratory tests routinely ordered by physicians and clinicians (4).

The situation is somewhat different for vitamin D. Although 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] is the biologically active form of the family that binds to the receptor, 25(OH)D is the parameter that is routinely measured to assess the vitamin D status of an individual. The conversion of 25(OH)D into 1,25$(OH)_2$D takes place in various cells and requires the transport of the monohydroxy form from the extracellular fluid inside the cell. Two transport mechanisms are mainly involved: one is based on the receptor megalin and relies on the total concentration of 25(OH)D; the other is the passive diffusion of the free ligand through the cell membrane and involves the concentration of free 25(OH)D (5). This tiny fraction is in equilibrium with the forms bound to VDBP and to albumin and is consequently dependent on the levels of both binding proteins. Whether the biological activity of vitamin D is more closely linked to the concentration of total or free 25(OH)D depends, therefore, on the predominant transport mechanism that is involved in each tissue and organ.

Although methods for the quantification of total vitamin D metabolites have been ubiquitous since the 1970s, methods for the measurement of free vitamin D metabolites were first described in the 1980s and were based on centrifugal ultrafiltration (6, 7). Briefly, a diluted sample was spiked with tritium-labeled 25$(OH)D_3$ or 1,25$(OH)_2D_3$ and with [^{14}C] glucose, and then placed in the ultrafiltration device and centrifuged. The measurement of ^{3}H and ^{14}C, both in the sample residue and in the ultrafiltrate, made it possible to calculate the percentage of the free vitamin D metabolite. Total 25(OH)D or 1,25$(OH)_2$D concentrations were obtained by classical methods and subsequently led to the concentrations of the related free forms. This method was very useful in providing preliminary evidence of the importance of measuring free vitamin D metabolites but suffered several drawbacks. Several assumptions were made and the technique proved to be cumbersome and highly time- and resource-consuming. Moreover, two assays had to be performed and the laboratory had to be equipped for the manipulation of both ^{3}H and ^{14}C. For these reasons, this method has not been extensively used,

Guest edited as a special report on "The Importance of Vitamin D Assay Standardization and the Vitamin D Standardization Program" by Stephen P.J. Brooks and Christopher T. Sempos.

Corresponding author's e-mail: nicolas.heureux@diasource.be

DOI: 10.5740/jaoacint.17-0084

and further research has been conducted using calculation algorithms.

Calculation of free 25(OH)D requires the measurement of three or four parameters (8, 9). At a minimum, both binding proteins (VDBP and albumin) and total 25(OH)D have to be assayed. Ideally, the genotyping of VDBP should also be performed in order to select the correct binding coefficient to be used in the equation (8, 9). Recent studies have shown contradictory findings regarding the VDBP binding coefficient (10, 11). This methodology has been extensively used over the past 10 years (12–15). However, the accuracy of some of the VDBP assays has been questioned (10). VDBP exists in different forms, and the assays that are based on monoclonal antibodies are too specific toward certain isoforms and tend to underestimate the binding protein concentration in some of the samples. Therefore, several research groups have called for a direct measurement of free 25(OH)D.

Recently an ELISA was developed for the direct measurement of free 25(OH)D (16, 17).

METHOD

The ELISA described here is a quantitative immunoassay for in vitro determination of the concentration of free 25(OH)D in serum.

Principles of the Method

The free 25(OH)D ELISA is based on a two-step immunoassay procedure performed in a microtiter plate. During the first incubation step, free 25(OH)D [25(OH)D_2 and 25(OH)D_3] is bound to the anti-vitamin D antibody coated on the wall of the microtiter plate. The in vivo equilibrium between free and bound 25(OH)D is minimally disturbed. After washing, a fixed amount of biotinylated 25(OH)D is added to each well. The nonbound biotinylated 25(OH)D is removed by washing, and a streptavidin peroxidase conjugate is added. In the next step, 3,3′,5,5′-tetramethylbenzidine (TMB) chromogenic substrate is added. Lastly, the reaction is stopped by adding stop reagent, and the absorbance at 450 nm is read using a plate spectrophotometer. The concentration of free vitamin D in the sample is inversely proportional to the absorbance.

Reagents

(a) Anti-vitamin D antibody–coated plate, coated with a mouse anti-25(OH)D_2/D_3 monoclonal antibody (18). The plate is composed of 96 wells of 350 µL capacity each.

(b) Sample diluent containing a surfactant and the biocide ProClin™.

(c) Concentrated biotinylated 25(OH)D in preservation buffer.

(d) Streptavidin-peroxidase conjugate solution containing ProClin.

(e) Calibrators A–F based on depleted human serum and containing ProClin, 5-bromo-5-nitro-1,3-dioxane, and increasing concentrations of 25(OH)D.

(f) Controls 1 and 2 based on normal human serum and containing ProClin and 5-bromo-5-nitro-1,3-dioxane.

(g) Concentrated wash buffer containing ProClin.

(h) Enhanced TMB substrate.

(i) 1 M HCl as stop solution.

(j) Biotinylated 25(OH)D dilution buffer containing ProClin and 5-bromo-5-nitro-1,3-dioxane.

Protocol

(a) Reagents are prepared according to the assay instructions. Lyophilized calibrators and controls are reconstituted, and the working dilution of the biotinylated 25(OH)D reagent is prepared prior to use. The required number of strips are removed from the Stripwell plate.

(b) A 90 µL aliquot of sample diluent is added to each well.

(c) A 10 µL aliquot of reconstituted calibrator, control, or sample is transferred in duplicate into the appropriate wells of the Stripwell plate.

(d) The plate is incubated for 90 min at 37°C, with shaking.

(e) The plate is washed three times with 350 µL wash buffer.

(f) A 100 µL aliquot of the working dilution of the biotinylated 25(OH)D reagent is added to the wells.

(g) The plate is incubated for 30 min at 37°C, with shaking.

(h) The plate is washed three times with 350 µL wash buffer.

(i) A 100 µL aliquot of the streptavidin-peroxidase reagent is added to each well.

(j) The plate is incubated for 20 min at 37°C, with shaking.

(k) The plate is washed three times with 350 µL wash buffer.

(l) A 100 µL aliquot of TMB substrate reagent is added to each well.

(m) The plate is incubated for 15 min at room temperature, stationary and protected from light.

(n) A 100 µL aliquot of stop reagent is added to each well.

(o) The adsorption is read at 450 nm within 5 min.

Typical Data

Typical optical densities obtained for each calibrator of the assay are shown in Table 1.

Performance Data

All testing was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines (19). The following equipment was used:

(a) 3-Cabin Thermo Scientific iEMS Microplate Incubator/Shaker.

(b) BioTek ELx50 eight-well microplate washer.

(c) BioTek ELx800 plate reader (dynamic range 0–3.0 OD).

(d) IVDfit software version 1.11 for four-parameter logistic curve fitting without weighting.

Table 1. Typical optical densities obtained for each calibrator of the assay

Calibrator	Concn, pg/mL	Avg. absorbance at 450 nm
A	0.0	2.004
B	3.3	1.446
C	7.7	1.019
D	13.1	0.720
E	23.6	0.417
F	40.3	0.226

Table 2. Precision CV values of calibrators, assay controls, and serum sample pools[a]

Sample type	Concn, pg/mL	CV, %
Calibrator B	5.0	6.7
Calibrator C	6.2	6.0
Calibrator D	7.7	5.0
Calibrator E	12.9	3.7
Control 1	7.6	6.7
Control 2	11.0	5.0
Serum sample pool 1	6.0	10.2
Serum sample pool 2	10.9	7.6
Serum sample pool 3	24.9	5.5

[a] CV values were calculated from the precision study based on the CSLI guideline EP05-A3.

Precision

Precision of the assay (Table 2) was evaluated on serum sample pools 1–3, on assay calibrators B–E, and on controls 1 and 2 during a 20-day study, based on CLSI guideline EP05-A3 (19). All samples were aliquoted and appropriately stored to ensure equivalence of the sample quality in each run. The serum sample pools were distributed along the calibration curve range. During 21 days, two runs were performed each day, with 2 hours between runs. The order of the serum sample pools was different in the morning and afternoon runs. Serum sample pools, assay calibrators, and controls were measured in duplicate. The dose of the serum sample pools was calculated using a four-parameter logistic-fit procedure.

The precision was further evaluated in a study comparing the performances of the assay when run manually and on a Dynex DS2 platform (Table 3). Precision was evaluated over 20 days, with two runs each day. One run was performed in the morning and one in the afternoon.

Sensitivity

The limit of blank (LOB) and LOD were determined in a study comparing the performances of the assay when run manually and on a Dynex DS2 platform (Table 4).

The LOB was determined by measuring 38 replicates of a vitamin D–depleted serum (<0.5 pg/mL) over 20 days. LOB calculation was done using the formula LOB = the result at position [N (p/100) + 0.5], i.e., non-Gaussian distribution, where N = the number of samples measured; and p = the 95th percentile.

The LOD was determined by measuring 38 replicates of four low-concentration samples over 20 days. The LOD was calculated using the formula LOD = LOB + Cp × SDp, where

Table 3. Precision CV values obtained when performing the assay manually versus on an automated ELISA platform (Dynex DS2)

ELISA type	Assay control	Concn, pg/mL	CV, %
Manual	Control 1	7.4	5.8
	Control 2	12.9	4.4
Dynex DS2	Control 1	6.7	4.2
	Control 2	11.2	3.7

Table 4. Sensitivity values (LOB and LOD) calculated when performing the assay manually versus on an automated ELISA platform (Dynex DS2)

ELISA	LOB, pg/mL	LOD, pg/mL
Manual	0.96	1.83
Dynex DS2	0.52	1.28

Cp = 1.645 ÷ [1 – 1/(4 × f)]; f = the total number of LOD determinations (in this case 38 × 4); and SDp = the pooled SD for all measurements.

Accuracy

Accuracy was evaluated in 15 samples by comparing the results obtained in the ELISA with the results obtained using a dialysis method. Dialysis has been used in the measurement of free hormones for many years (20). In a standard-rate dialysis assay, two chambers are separated by a dialysis membrane. The MW cutoff of this membrane was chosen so that it retained the bound fraction. Undiluted sample was added to a tube containing tritium vitamin D tracer, and the tube was incubated for 15 min at 37°C. The sample containing the tritium vitamin D tracer was transferred to the dialysis cassette, and the same volume of neat sample was added to the other side of the dialysis cassette. The cassette was incubated overnight at 37°C, after which the samples were removed from the dialysis cassettes. The radioactivity of the samples was measured, and the percentage of free 25(OH)D was calculated from the counts. In addition, total 25(OH)D in the samples was measured by LC-MS.

Free 25(OH)D was calculated from the percentage obtained by the dialysis experiment and from the total 25(OH)D (Figure 1).

Cross-Reactivity

Cross-reactivity of 25(OH)D_2 in the free 25(OH)D assay cannot simply be determined using the standard procedure

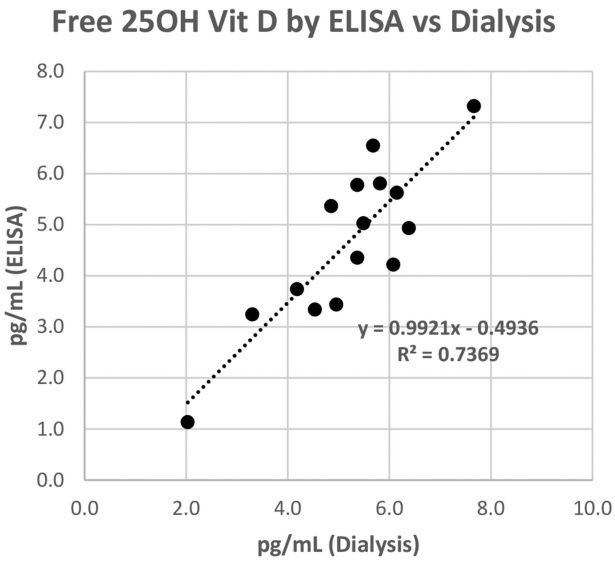

Figure 1. The free 25(OH)D ELISA was compared with the dialysis technique; 15 samples were measured in both methods and a linear regression was established.

Table 5. Recovery of free 25(OH)D after spiking serum samples with VDBP or albumin

		Concn in spiked sample, pg/mL		
Sample No.	Concn in sample, pg/mL	VDBP spike (2 mg/mL)	Albumin spike (60 mg/mL)	Recovery, %
1	9.7	3.5		36
2	8.1	3.1		38
3	12.0	4.2		35
4	17.9		9.4	53
5	10.0		5.8	58
6	10.9		6.1	56

for determining cross-reactivity. Differences in the binding of $25(OH)D_2$ and $25(OH)D_3$ have to be taken into account. Therefore, the cross-reactivity of the antibody was determined in a total 25(OH)D assay using the same antibody by comparing the effect of $25(OH)D_2$ addition with that of $25(OH)D_3$ addition in a serum sample, at three different concentrations. The recovery of each metabolite was calculated for the three spiking concentrations, and the cross-reactivity was established by comparing the recovery of both 25(OH)D forms.

The mean cross-reactivity of $25(OH)D_2$ when added to a donor serum was 77%.

Addition of Binding Proteins

The effect of adding VDBP and human serum albumin was determined in the sera of three different donors. Each serum sample was spiked with either 2 mg/mL VBDP or 60 mg/mL albumin and tested in triplicate using the free 25(OH)D ELISA. The recovery was then calculated for each sample by comparing the concentration obtained after spiking to the concentration of the neat serum (Table 5). The chosen concentrations for spiking were within the range of normal concentrations for each protein (10, 21).

Discussion

25(OH)D is tightly bound in serum to VDBP and moderately bound to albumin. The bound form is in equilibrium with the free form, although the equilibrium is largely shifted toward the protein-bound fraction due to the high-affinity binding of 25(OH)D to VBDP. The free 25(OH)D represents only about 0.04% of the total 25(OH)D concentration, depending on the VDBP levels.

In this ELISA, the best results were obtained when the incubation buffer (also designated as sample diluent) and the serum sample were incubated in the coated wells at a ratio of 9:1 for 90 min at 37°C with vigorous shaking. After three washing steps, only the captured free 25(OH)D remained in the assay wells, and a classical ELISA protocol was performed. Because of the extremely low concentrations of free 25(OH)D (typically 2–8 pg/mL), an ultrasensitive ELISA was developed.

Typical data show the relationship between the concentration of the assay calibrators and the obtained optical densities. The highest calibrator was set at around 40 pg/mL in order to cover any healthy or pathological sample concentration.

The precision of the assay was determined in two different studies. The first was performed according to the corresponding CSLI guideline, whereby the total precision of the assay was calculated on the calibrators, on two assay controls, and on three serum sample pools. As can be expected for a competitive immunoassay, the imprecision of the results increased at lower concentrations, and vice versa. The CVs obtained at physiological concentrations ranged between 5 and 10%, which is within the normal range of a competitive ELISA. The second study compared the precision obtained when the assay was performed manually and on an automated ELISA platform. The automated liquid handling system proved to be slightly superior to the manual operator, and CVs below 5% were obtained at the two concentrations studied.

To evaluate the sensitivity of the assay, the LOB and the LOD were evaluated both manually and using an automated ELISA processor, according to CLSI guideline EP17-A2. The precision of the automated ELISA processor was better than the precision obtained with the manual procedure, resulting in better analytical sensitivity. However, the sensitivity obtained through manual operations was more than acceptable and typically below the lowest concentrations observed in patient samples.

The accuracy, or trueness, of immunoassays can typically be evaluated by comparing the results obtained with the assay to the results obtained by a reference measurement procedure (RMP). It is unfortunate that there is no RMP for free 25(OH)D. Therefore, rate dialysis was selected as the method for comparison. Rate dialysis is regarded as a reference method for the measurement of free hormones, although it is completely satisfactory (22). The linear regression established between the two methods provided a slope of nearly 1.00, an intercept of −0.49 pg/mL, and an R^2 value of 0.74, confirming that the method is correctly calibrated and provides accurate results.

The cross-reactivity profile of immunoassays is commonly determined by spiking samples with increasing concentrations of cross-reactants and by comparing the results obtained for the spiked and nonspiked samples. Vitamin D assays in general are prone to deleterious influences of spiking with exogenous substances, and this method of determining cross-reactivity profiles is increasingly regarded as inappropriate. For a free 25(OH)D assay, this is even more problematic because the spiked vitamin D metabolites will interfere with the binding proteins to different levels and will strongly disturb the equilibrium between the bound and unbound forms of 25(OH)D. For this reason, the cross-reactivity of the antibody used in this assay was evaluated in a total 25(OH)D assay. The obtained results are a good indication of the cross-reactivity of the antibody in the free 25(OH)D assay because the antibody is mainly responsible for the cross-reactivity observed. In this case, the monoclonal antibody used was found to cross-react with $25(OH)D_2$ at 77%.

Additional validation experiments included the addition of VDBP or albumin to serum samples. Increasing concentrations of binding proteins should decrease the concentration of free 25(OH)D, and this is what was observed. The concentrations of VDBP and albumin added to the patient samples were within the physiological range.

The free 25(OH)D ELISA has been used by several research groups that have studied the variability of free 25(OH)D levels in clinical populations (23), compared the results obtained with calculation methods (8, 24, 25), investigated the concentration of total and free 25(OH)D in cirrhotic patients with and without synthetic dysfunction (25), reviewed the vitamin D paradox in African Americans (26), looked at the differential responses

of free 25(OH)D to vitamin D_2 and vitamin D_3 in mice (27), considered the response of free 25(OH)D concentration to vitamin D_3 administration in older adults without sun exposure (28), examined the relationship between free 25(OH)D and atopy and measures of pulmonary function in Peruvian children with asthma (29), considered free 25(OH)D in obesity (30), and inspected the effects of VDBP phenotypes and vitamin D supplementation on free 25(OH)D (31).

Conclusions

Free 25(OH)D is currently being investigated by the research community for its potential value as a better marker of vitamin D status than total 25(OH)D. With different groups calling for a direct measurement method, the development of an ELISA comes at an opportune time. The ELISA is based on monoclonal anti-25(OH)D antibodies and uses a specific incubation buffer that enables the capture of the free fraction of 25(OH)D only. The assay has been analytically validated in terms of precision, accuracy, sensitivity, and specificity. Additional validation work is currently being conducted, including a multicenter reproducibility study.

The free 25(OH)D ELISA can be used as a valuable tool in studies to establish the clinical relevance of free 25(OH)D.

References

(1) Heureux, N. (2017) *Adv. Clin. Chem*., **78**, 59–101.

(2) Stockigt, J. (2011) Clinical Strategies in the Testing of Thyroid Function, Endotext, MDText.com, Inc., South Dartmouth (MA)

(3) Mendel, C.M. (1989) *Endocr. Rev.* **10**, 232–274. doi:10.1210/edrv-10-3-232

(4) Trost, L.W., & Mulhall, J.P. (2016) *J. Sex. Med.* **13**, 1029–1046.

(5) Hewison, M. (2010) in *Nuclear Receptors*, Proteins and Cell Regulation Series, Vol. 8, C.M. Bunce and M.J. Campbell (Eds.), Springer International Publishing, Basel, Switzerland, pp 381–417

(6) Bikle, D.D., Gee, E., Halloran, B., & Haddad, J.G. (1984) *J. Clin. Invest.* **74**, 1966–1971. doi:10.1172/JCI111617

(7) Bikle, D.D., Halloran, B.P., Gee, E., Ryzen, E., & Haddad, J.G. (1986) *J. Clin. Invest.* **78**, 748–752. doi:10.1172/JCI112636

(8) Schwartz, J.B., Lai, J., Lizaola, B., Kane, L., Markova, S., Weyland, P., Terrault, N.A., Stotland, N., & Bikle, D. (2014) *J. Clin. Endocrinol. Metab.* **99**, 1631–1637. doi:10.1210/jc.2013-3874

(9) Johnsen, M.S., Grimnes, G., Figenschau, Y., Torjesen, P.A., Almås, B., & Jorde, R. (2014) *Scand. J. Clin. Lab. Invest.* **74**, 177–183 [erratum, http://dx.doi.org/10.3109/00365513.2014.926094]. doi:10.3109/00365513.2013.869701

(10) Nielson, C.M., Jones, K.S., Chun, R.F., Jacobs, J.M., Wang, Y., Hewison, M., Adams, J.S., Swanson, C.M., Lee, C.G., Vanderschueren, D., Pauwels, S., Prentice, A., Smith, R.D., Shi, T., Gao, Y., Schepmoes, A.A., Zmuda, J.M., Lapidus, J., Cauley, J.A., Bouillon, R., Schoenmakers, I., & Orwoll, E.S. (2016) *J. Clin. Endocrinol. Metab.* **101**, 2226–2234. doi:10.1210/jc.2016-1104

(11) Denburg, M.R., Hoofnagle, A.N., Sayed, S., Gupta, J., de Boer, I.H., Appel, L.J., Durazo-Arvizu, R., Whitehead, K., Feldman, H.I., & Leonard, M.B. (2016) *J. Bone Miner. Res.* **31**, 1128–1136. doi:10.1002/jbmr.2829

(12) Koenig, K.G., Lindberg, J.S., Zerwekh, J.E., Padalino, P.K., Cushner, H.M., & Copley, J.B. (1992) *Kidney Int.* **41**, 161–165. doi:10.1038/ki.1992.22

(13) Brown, A.J., & Coyne, D.W. (2012) *Kidney Int.* **82**, 5–7 doi:10.1038/ki.2012.135

(14) Jeng, L., Yamshchikov, A.V., Judd, S.E., Blumberg, H.M., Martin, G.S., Ziegler, T.R., & Tangpricha, V. (2009) *J. Transl. Med.* **23**, 7–28

(15) Powe, C.E., Evans, M.K., Wenger, J., Zonderman, A.B., Berg, A.H., Nalls, M., Tamez, H., Zhang, D., Bhan, I., Karumanchi, S.A., Powe, N.R., & Thadhani, R. (2013) *N. Engl. J. Med.* **369**, 1991–2000. doi:10.1056/NEJMoa1306357

(16) DiaSource, Free 25OH Vitamin D, ELISA, 96 Tests (RUO), http://www.diasource-diagnostics.com/RUO-Products/ImmunoAssays/Bone-Metabolism/Vitamin-D/Free-25OH-Vitamin-D-ELISA-96-tests-RUO (accessed on January 13, 2017)

(17) Future Diagnostics Solutions, Manufacturing, http://future-diagnostics.com/assay-manufacturing-services/the-products/ (accessed on January 13, 2017)

(18) Anciaux, M., Mathieu, F., Lin, F., & Poncelet, M. (2013) Process for the production of a hybridoma and antibody obtained therefrom, able to recognize more than one vitamin D metabolite. Patent No. EP2316854 B1, http://www.google.com/patents/EP2316854B1?cl=en

(19) McEnroe, R.J., Durham, A.P., Goldford, M.D., Kondratovich, M.V., Lababidi, S., Magari, R., Middle, J.G., Pierson-Perry, J.F., & Vaks, J.E. (2014) *Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline—Third Edition*, CLSI document EP05-A3, Clinical and Laboratory Standards Institute, Wayne, PA

(20) Ekins, R. (1992) *Clin. Chem.* **36**, 1289–1293

(21) Devaraj, S., & Wheeler, T.M. (2015) Albumin, Reference Range, http://emedicine.medscape.com/article/2054430-overview (accessed on January 13, 2017)

(22) Marshall, W.J., & Bangert, S.K. (Eds) (2008) *Clinical Biochemistry: Metabolic and Clinical Aspects*, 2nd Ed., reprint, Elsevier Ltd, Churchill Livingstone, Edinburgh, United Kingdom

(23) Schwartz, J.B., Lai, J., Lizaola, B., Kane, L., Weyland, P., Terrault, N.A., Stotland, N., & Bikle, D. (2014) *J. Steroid Biochem. Mol. Biol.* **144**, 156–158. doi:10.1016/j.jsbmb.2013.11.006

(24) Nielson, C.M., Jones, K.S., Bouillon, R., Chun, R.F., Jacobs, J., Wang, Y., Hewison, M., Adams, J.S., Swanson, C.M., Lee, C.G., Vanderschueren, D., Pauwels, S., Prentice, A., Smith, R.D., Shi, T., Gao, Y., Zmuda, J.M., Lapidus, J., Cauley, J.A., Schoenmakers, I., & Orwoll, E.S. (2016) *N. Engl. J. Med.* **374**, 1695–1696. doi:10.1056/NEJMc1513502

(25) Lai, J.C., Bikle, D.D., Lizaola, B., Hayssen, H., Terrault, N.A., & Schwartz, J.B. (2015) *Liver Int.* **35**, 2294–2300. doi:10.1111/liv.12819

(26) Aloia, J., Mikhail, M., Dhaliwal, R., Shieh, A., Usera, G., Stolberg, A., Ragolia, L., & Islam, S. (2015) *J. Clin. Endocrinol. Metab.* **100**, 3356–3363. doi:10.1210/JC.2015-2066

(27) Shieh, A., Chun, R.F., Ma, C., Witzel, S., Meyer, B., Rafison, B., Swinkels, L., Huijs, T., Pepkowitz, S., Holmquist, B., Hewison, M., & Adams, J.S. (2016) *J. Clin. Endocrinol. Metab.* **101**, 3070–3078. doi:10.1210/jc.2016-1871

(28) Schwartz, J.B., Kane, L., & Bikle, D. (2016) *J. Am. Geriatr. Soc.* **64**, 65–72. doi:10.1111/jgs.13774

(29) Pollard, S.L., Lima, J.J., Romero, K., Mougey, E.B., Tarazona-Meza, C.E., Tomaino, K., Malpartida Guzman, G., Hansel, N.N., & Checkley, W. (2016) *Am. J. Resp. Crit. Care Med.* **193**, A2764

(30) Walsh, J.S., Evans, A.L., Bowles, S., Naylor, K.E., Jones, K.S., Schoenmakers, I., Jacques, R.M., & Eastell, R. (2016) *Am. J. Clin. Nutr.* **103**, 1465–1471. doi:10.3945/ajcn.115.120139

(31) Sollid, S.T., Hutchinson, M.Y., Berg, V., Fuskevåg, O.M., Figenschau, Y., Thorsby, P.M., & Jorde, R. (2016) *Eur. J. Endocrinol.* **174**, 445–452. doi:10.1530/EJE-15-1089