CDC VDSCP- Total 25hydroxy Vitamin D Certified Procedures (UPDATED 05/2014)

The following laboratories have successfully passed the performance criterion of ±5% mean bias to the CDC and University of Ghent Vitamin D2 and D3 Reference Method and an overall imprecision of ≤10% over the concentration range of 22-275 nmol/L for total 25hydroxy vitamin D. Laboratories are awarded Certificates for successfully completing bias and imprecision testing using specific methods, reagent lots, calibrator lots and instrumentation. During the year it is the responsibility of the participant to insure that the results of their method remain consistent over time, throughout the year, between lots, and over the measurement range reported. It is not the intent of the CDC VDSCP to certify each lot of reagents and beyond the measurement range of 22-275 nmol/L at this time.

Participant	**Measurement Principle**	**Method Identifier***	**Measurement Range (nmol/L)**	**Participants contact information**	**Certification Date** *(active for 1 year)*
Endocrine Sciences Laboratory, LabCorp	LC/MS/MS†	Test Code 500510 and 500116 fractionated 25-OH Vitamin D	2.4-625	Dr. Walt Chandler Chandld@labcorp.com (800)444-9111	Feb 2014
Department of Clinical Chemistry, University of Leige, CHU Sart-Tilman, Liege, Belgium	LC/MS/MS†	Serum Total Vitamin D	5-625	Prof. Etienne Cavalier etienne.cavalier@chu.ulg.ac.be +32 4 3668822	Feb 2014
Mayo Medical Laboratories	LC/MS/MS†	Serum 25 hydroxyvitamin D2 and D3	15-740	Larry Dodge Dodge.larry@mayo.edu (507)-538-0400 Eric Bro Bro.eric@mayo.edu (507)266-4348	Feb 2014
Siemens Healthcare Diagnostics	Chemiluminescence Immunoassay	ADVIA Centaur® Vitamin D Total assay	10.5-375	James Freeman james.freeman@siemens.com 914-524-2902	Feb 2014
Pathology Associates Medical Laboratory, LLC	LC/MS/MS†	VITAMIN D2 D3, 25-HYDROXY BY LC/MS/MS	10.0-300	Dr. Carmen Wiley Carmen.wiley@paml.com (509_755-8554	Feb 2014
Immunodiagnostic Systems	Immunoassay	IDS-iSYS 25-Hydroxy Vitamin D	15.0-315	Heather Pham heather.pham@idsplc.com (877)-852-6210	Feb 2014
Immunodiagnostic Systems	Immunoassay	25-Hydroxy Vitamin D EIA	0-400	Heather Pham heather.pham@idsplc.com (877)-852-6210	Feb 2014
Vitamin D Research Group University College Cork	LC/MS/MS†	Total serum 25OHD	1.99-254	Prof. Kevin Cashman k.cashman@ucc.ie +353 21 4901317	Feb 2014
Douglas Hanly Moir Pathology	LC/MS/MS†	Serum 25 hydroxyvitamin D2 and D3	4-600	Dr Grahame Caldwell gcaldwell@dhm.com.au +61 298555380 Andy Liu aliu@dhm.com.au +61 298555275	Feb 2014

* Method Identifier is an internal code used to represent the standardized method used by the laboratory or manufacturer.

† LC/MS/MS – Liquid Chromatography Tandem Mass Spectrometry

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IMPROVING HEALTHCARE THROUGH LABORATORY MEDICINE

SERIES

Vitamin D

Progress Toward Standardization

By James Freeman and Kimberly Wilson

In the past decade, interest in the steroid hormone vitamin D has soared as researchers have uncovered links between vitamin D and conditions such as heart disease, diabetes, rheumatoid arthritis, multiple sclerosis, Parkinson's disease, and some cancers (*1*). With the news of the connection to these highly prevalent diseases, clinical laboratories quickly experienced a surge in demand for vitamin D testing.

Not surprisingly, in vitro diagnostic manufacturers responded by introducing a number of new immunoassays for measuring 25-hydroxyvitamin D. Such assays offer laboratories a means to automate testing in order to meet the increased demand. Laboratories also have developed their own methods, such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), to measure vitamin D.

However, as test results accumulated, clinical laboratory professionals observed that vitamin D assays from different commercial sources and platforms produced inconsistent results from the same patient specimen. In some instances, these differences were large enough to affect whether a patient would be classified as having sufficient or deficient vitamin D levels.

To correct these disparities, the Vitamin D Standardization Program (VDSP), an initiative of the National Institutes of Health Office of Dietary Supplements (NIH ODS), was launched in 2010 in collaboration with the National Institutes of Health, the Centers for Disease Control and Prevention (CDC), the National Institute for Standards and Technology (NIST), and Ghent University in Belgium. This article will describe the progress toward standardization of vitamin D assays and what laboratories should do until then to ensure patient safety.

Vitamin D Biochemistry

Vitamin D is a steroid hormone involved in regulating calcium homeostasis in part through intestinal absorption of calcium and renal re-absorption of calcium. Often referred to as the "sunshine vitamin", vitamin D is actually a fat-soluble hormone that the body can synthesize. There are two major types: vitamin D2 (ergocalciferol), which is synthesized by plants and is not produced by the human body; and vitamin D3 (cholecalciferol), which is made by bare skin when exposed to sunlight.

Whether it is absorbed through unprotected skin or ingested then absorbed by the intestines, vitamin D binds to two proteins in the bloodstream, albumin and vitamin D binding protein, and then travels to the liver. From there it undergoes two hydroxylations. In the liver, it is transformed into 25-hydroxyvitamin D or calcidiol, which is the primary circulating form of vitamin D and the most commonly measured form in serum. In the kidneys, it is transformed into 1,25 dihydroxyvitamin D or calcitriol, which is the biologically active form of vitamin D.

As levels of 25-hydroxyvitamin D reflect vitamin D synthesized through unprotected skin or ingested in food or supplements and absorbed by the intestines, its serum concentration is recognized as the best indicator of an individual's vitamin D status. While not biologically active like 1,25 dihydroxyvitamin D, the former has a much longer half-life (2–3 weeks versus 4–6 hours) and is not influenced by changes in calcium and parathyroid hormone levels (*2*).

Technical Challenges

Even though 25-hydroxyvitamin D is the preferred vitamin D analyte, measuring it is not straightforward. In serum, the hormone is completely bound to proteins. Furthermore, the normal protein levels in human serum are capable of binding hundreds of nanograms of vitamin D. To measure vitamin D in blood, it must first be released from its binding protein, which has a high affinity constant on the order of $1 \times 10^7 - 10^9$.

For LC-MS/MS analysis methods, labs first precipitate the binding protein from serum, followed by organic extraction of the vitamin D from the sample and then analysis. Of particular importance, most LC-MS/MS methods cannot distinguish the C3 epimer, 3-epi-25-hydroxyvitamin D, from 25-hydroxyvitamin D. The biological importance of the former molecule is not known; however, its inclusion in the measurement could contribute to falsely high levels of vitamin D. In some instances, this elevation could make a patient who is vitamin D deficient appear normal, or in more extreme situations, the patient may appear abnormally high, approaching toxic levels (*3–6*).

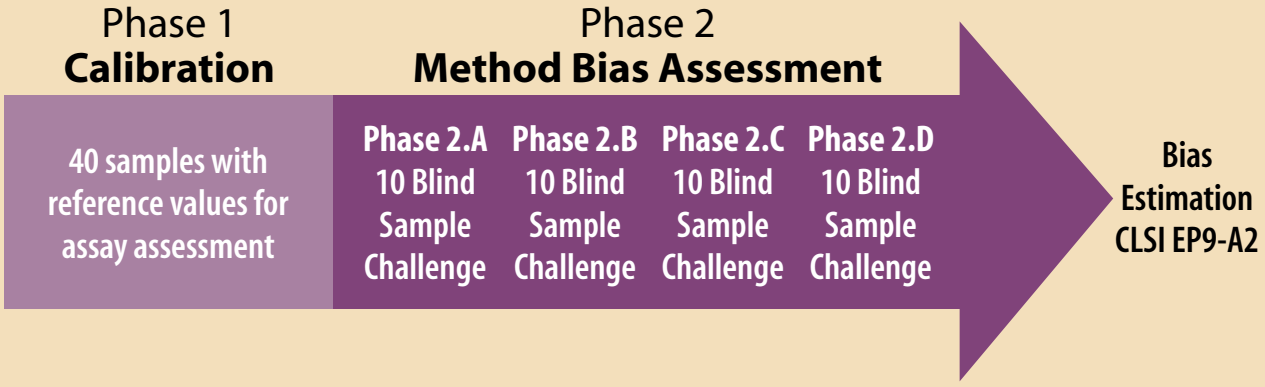
With commercially available immunoassays, each manufacturer has its own proprietary process to separate vitamin D from its binding protein. Newer assays on the market also contain specific blockers to minimize the interference of heterophilic antibodies in patient samples that may

bind the detection antibody and produce erroneous results. This type of reagent is especially important for reducing the occurrence of low or high outliers. When assays do not account for heterophilic antibody interferences, the correlation coefficient between methods may be lower.

In 2010, a panel of 25 vitamin D experts from various clinical fields recommended using vitamin D assays that measure both 25-hydroxyvitamin D2 and vitamin D3 so that dietary supplementation from both plant and animal sources would be measureable (*7*). Therefore, laboratories ideally need a test that can recognize both forms, not just one.

Figure 1

Phases of the CDC HoSt Program



Courtesy of CDC.

The Disparity in Vitamin D Assays

Today, however, due to the lack of an internationally recognized, commutable vitamin D reference material, discrepancies exist across testing methods for vitamin D, as well as among commercially available vitamin D tests. Comparisons of manufacturers' assays and laboratory-developed assays also show scatter in test results that may be due to differences in the way the assays measure vitamin D, different methods to release vitamin D from its binding proteins, and interference from heterophilic antibodies.

The observed variability in vitamin D assays is not unique. As laboratory scientists know well, a number of factors may cause differences among commercial assays that measure lipids, hemoglobin A1c, and hormones. Such variability arises from the complex nature of these analytes in human serum, the assay method, and antibody specificity.

Furthermore, steroids such as vitamin D typically are bound to proteins—often very tightly—and must be released before the immunoassay reaction can take place. Differences in the releasing reagents used also can lead to differences in patient sample results between different assays.

Antibody specificity, especially for vitamin D, also plays an important role in understanding the differences between assay results. For example, a specimen tested with an assay that measures only 80% of vitamin D2 relative to vitamin D3 will produce a different result when tested with an assay that measures 100% of both vitamin D2 and vitamin D3.

The quality and source of materials that manufacturers use to standardize an assay also may differ and contribute to the observed discrepancies. Manufacturers typically produce internal reference materials to assign master curve standards, but use other calibration materials for patient test results. This practice may contribute to differences in assay results between manufacturers.

The Vitamin D Standardization Program

To minimize assay-to-assay variability for vitamin D, a reference material is needed to standardize assays and eventually harmonize patient results. Reference standards serve as anchors that can provide comparability across time and methods. But developing an international reference material is not easy, and success depends on that material being commutable across all manufacturers and methods.

The VDSP has developed protocols for standardizing procedures to measure 25-hydroxyvitamin D in National Health/Nutrition Surveys. The group also aims to improve public health practice by promoting measurements that are accurate and comparable over time, across locations, and independent of laboratory methods. The CDC has introduced a Vitamin D Standardization-Certification Program to ensure reliable clinical vitamin D measurement.

The Importance of Standardization

International standardization programs have already been successful in standardizing lipid measurements, immunoassays for hormones such as estradiol and testosterone, and hemoglobin A1C. Critical factors in establishing successful standardization programs include minimizing matrix effects in the standard reference material that may lead to spurious results, and maintaining commutability of standards across all manufacturers and all methods.

Over the last several years, multiple standardization certification programs sponsored by CDC have harmonized test results among manufacturers (*8*). For example, the Lipid Standardization Program provides accuracy-based standards for measuring total cholesterol, triglycerides, high-density lipoprotein cholesterol, apolipoprotein A-I, and apolipoprotein B in U.S. and international laboratories.

The CDC Hormone Standardization (HoSt) Program is also aimed at introducing more accurate and precise steroid hormone tests for patient care, including testosterone, estradiol, and estrogen. It seems likely that the differences today between manufacturers' assays are likely the result of high concentrations of steroid-binding proteins present in serum, a situation similar to that of vitamin D.

In fact, the HoSt Program has begun work on vitamin D as the next hormone assay to be part of the program. Phase 1 of the program has made 40 native patient samples available that have been assayed by isotope dilution, liquid chromatography tandem mass spectrometry (ID-LC-MS/MS), the same method used to establish vitamin D values for the VDSP. The Joint Committee for Traceability in Laboratory Medicine (JCTLM) also recognizes ID-LC-MS/MS as the reference method for vitamin D, and it is comparable with other reference methods, such as the NIST reference method. Manufacturers will use these 40 samples to make adjustments to their own internal standard materials, if necessary, and to gain better alignment with the CDC certification process.

In the Absence of a Standard

Generally speaking, the ideal vitamin D standard material would be native human serum samples representing endogenous levels of vitamin D over a range of 5–150 ng/mL. For some other immunoassays, manufacturers have used reference standards from the World Health Organization (WHO) to standardize their assays. Unfortunately, a WHO reference material is not available for vitamin D.

Instead, manufacturers have used the NIST standard reference material (SRM) 972, which has been available since 2009 and represents the first step in vitamin D standardization. Together with the U.K. Vitamin D External Quality Assurance Scheme (DEQAS), this reference material has been helpful in improving the accuracy of LC-MS/MS analyses across laboratories. In fact, imprecision in LC-MS/MS methods demonstrated in the DEQAS surveys has greatly decreased over time as the calibration has been adjusted to bring individual laboratories closer to the all laboratory trimmed mean.

Although SRM 972 is suitable for LC-MS/MS methods, only Level 1 at 23.9 ng/mL is suitable for immunoassays. The other levels are adulterated and may cause matrix effects in immunoassays. Some manufacturers have already made their immunoassays traceable to the LC-MS/MS method, but some variability can still occur based on the referenced LC-MS/MS.

Implementing the Vitamin D Standardization Program

Under the leadership of Christopher Sempos, PhD, of the NIH ODS, the VDSP partners began work on attempting to standardize 25-hydroxyvitamin D measurements across methods and manufacturers. They selected the NIST reference method procedure (RMP) as the primary reference method for measuring total 25-hydroxyvitamin D, including 25-hydroxyvitamin D2, 25-hydroxyvitamin D3, and 3-epi-25-hydroxyvitamin. A second method from Ghent University is also traceable to the NIST RMP.

Linda Thienpont, PhD, at Ghent University, has developed an ID-LC-MS/MS method for vitamin D in human serum that is traceable to NIST SRM 972 (*9*) and that separates the 3-epi-25-hydroxyvitamin D3 from 25-hydroxyvitamin D3. This analysis ensures that 25-hydroxyvitamin D3 is not overestimated in the result.

NIST researchers are working to create certified reference materials to decrease possible bias in the vitamin measurements (*10*). They have generated 50 unique patient specimens ranging in vitamin D concentration from 5.04–60 ng/mL, all with assigned values for the three vitamin D analytes using both established reference methods. These samples were distributed to manufacturers and select labs in December 2011. Because the patient reference samples are native, the researchers do not expect commutability issues.

Standardization of vitamin D measurement procedures is also part of the CDC HoSt program, conducted by the Clinical Chemistry Branch, Division of Laboratory Services, under the supervision of Hubert W. Vesper, PhD. In 2012, HoSt started to include vitamin D in its effort to improve the accuracy and comparability of hormone measurements.

The CDC Standardization Certification Program for Vitamin D is a two-phase process (Figure 1, above). Manufacturers and laboratories using laboratory developed tests to measure vitamin D receive 40 single-donor serum samples with value assignments for calibration purposes. Values are assigned by a JCTLM-recognized reference method; therefore, calibration using these samples allows manufacturers and laboratories to establish metrological traceability according to ISO 17511 (*11*). For immunoassays, participants receive a set of internal standards to match the patient reference samples. Once participants verify the calibration to the initial samples, CDC on a quarterly basis distributes

challenges with 10 blinded samples as part of phase two to verify and monitor stability of calibration over time.

When participants pass four consecutive surveys, they are awarded certification for 1 year (*12*). Renewal is an annual process, and the performance criteria for vitamin D are a 5% mean bias and 10% imprecision.

The Future of Vitamin D Results

Standardization of vitamin D assays is essential to patients' safety and health, but manufacturers and laboratories will need time to implement the results once they are available. Regulatory requirements will no doubt take time to be fully developed, and implementation by manufacturers will not occur immediately. So what can laboratories do until the standardized assays are available?

Accuracy-based testing systems, such as the NIST-NIH Vitamin D Metabolites Quality Assurance Program (VitDQAP), the College of American Pathologists (CAP), and DEQAS, bridge the gap in the standardization effort not covered by the CDC HoSt program by helping individual laboratories that cannot afford to participate in the program to standardize their measurements of total 25-hydroxyvitamin D and its component metabolites. Starting with the October 2013 round of DEQAS, NIST will assign values for all DEQAS materials used in the program.

Laboratories participating in the CDC Standardization Program Certificate will need to wait at least 1 year for their results to be released. In the meantime, external accuracy-based surveys such as VitDQAP, CAP, and DEQAS will take into consideration that participants may be at different stages of the standardization process when they submit results. It is equally important that laboratories are aware of this situation when interpreting these results.

Clearly, not only is the absence of an international vitamin D standard a concern for immunoassay manufacturers striving to produce assays that accurately measure vitamin D, but it is also a public health issue. Until we reach the goal of having standardized assays, researchers who publish study results in this area will likely continue to call for a vitamin D standard.

To be successful, standardization requires a collaborative process involving support of an independent international organization, selection of an official reference standard, and development of a commutable material. We are well on our way at this point. CLN

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