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CDC Standardization-Certification for Total 25-hydroxyvitamin D and Total Testosterone Assays

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Abstract

Variability in results of hormone assays such as total 25-hydroxyvitamin D and total testosterone has the potential to adversely affect proper patient care. Standardization of assays has been proposed as a way to address this problem. Assays that are standardized are designed to provide accurate results, traceable to "true" value-assigned certified reference materials and gold-standard reference methods. Results obtained using standardized methods can be compared across assays, institutions, populations, and with past and future test results, thereby improving diagnosis, treatment, and outcomes of patients.

Vitamin D is a hormone that is vital for healthy bones. Hypovitaminosis D leads to bone defects and has been associated with extraskeletal conditions and diseases. Currently, the concentrations of vitamin D (measured as 25[OH]D) in the circulation are largely determined using various commercialized automated methods; however, variability in results between methods has made it difficult to assign cutoff values for diagnosis and to develop public health guidelines. In 2010, an international collaborative effort, led by the Office of Dietary Supplements (ODS) of the U.S. National Institutes of Health (NIH), in collaboration with the National Institute of Standards and Technology (NIST), the Centers for Disease Control and Prevention (CDC), and Ghent University (Ghent, Belgium), established the CDC Vitamin D Standardization Program (VDSP) to address this problem.

The goals of the VDSP were to improve clinical and public health practice by developing reference methods and materials for standardization and harmonization of vitamin D assays so that results could be compared across manufacturers, laboratories, and time. Additionally, the CDC Vitamin D Standardization-Certification Program (VDSCP) was set up to maintain the quality of vitamin D assays through ongoing quarterly testing challenges of participant laboratories. On a quarterly basis, laboratories achieve certification if they meet CDC requirements of <10% imprecision and ±5% mean bias for the preceding four quarters.

Similar to the case for total vitamin D, inconsistencies in total testosterone results between assays have led to the CDC Hormone Standardization Program (CDC HoST) Certified Total Testosterone Procedures. The goal of the HoST project was to standardize testosterone measurements in order to improve patient care. Standardization of total testosterone measurements in serum is performed by determining whether the bias between CDC's value-assigned reference material and the laboratory values falls within predefined limits. Only then does CDC consider a laboratory standardized. On a quarterly basis, certification is granted if the HoST acceptance criterion of ±6.4% mean bias to the CDC Testosterone Reference Method is met for the most recent four quarters.

What Is Vitamin D?

Vitamin D is a group of steroid hormones that are essential for healthy bones and biological processes. The major forms are vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol), both available in the diet or as supplements (D represents the sum of D_3 and D_2). Vitamin D_3 (not vitamin D_2) can be formed in the skin by exposure to UVB sun rays. Both vitamin D_3 and D_2 forms circulate in blood and are hydroxylated in the liver to 25-hydroxycholecalciferol, 25(OH)D_3 and 25-hydroxyergocalciferol, 25(OH)D_2 and further hydroxylated in the kidney to form the biologically active molecules 1,25- dihydroxycholecalciferol D_3, 1,25$(OH)_2D_3$, (also known as calcitriol) and 1,25-dihydroxyergocalciferol, 1,25$(OH)_2D_2$. The active 1,25$(OH)_2$D mediates calcium absorption in the intestine and mineral release from bone (calcium, etc.) and stimulates calcium reabsorption in the kidney.

Although 1,25$(OH)_2$D is the biologically active form, total 25(OH)D is recognized as the best marker of vitamin D nutritional status.[1] This is because 25(OH)D is the most abundant vitamin D metabolite in blood (25[OH]D concentration is approximately 30 ng/mL vs. 1–5 ng/mL for 1,25$[OH]_2$D); is not as tightly regulated as 1,25$(OH)_2D_3$ in response to changes in calcium and parathyroid hormone concentrations; and is dependent on kidney function. In addition, 25(OH)D has a long half-life (around 3 weeks) compared to 1,25$(OH)_2$D (around 8 hours). Combined, these features make 25(OH)D relatively easy to measure. Importantly, oral 25(OH)D supplementation in patients was found to cure bone defects (rickets and osteomalacia) that are attributable to hypovitaminosis D. Currently, along with 25(OH)D, other potential metabolites with biological activity are being considered for standardization as supplemental markers. These markers include 1,25$(OH)_2$D; 3-epi-25(OH)D; 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$]; vitamin D-binding protein (DBP); free/bioavailable 25(OH)D; and PTH.[2]

Prevalence and Causes of Vitamin D Deficiency

Vitamin D deficiency and insufficiency are a global healthcare problem.[3] Approximately 30% of children and adults worldwide are vitamin D-deficient and 60% insufficient.[3,4] In the United States, 50% of children ages 1–5 and 70% of children ages 6–11 had a 25(OH)D <30 ng/mL.[3,5] The causes of vitamin D deficiency include lack of sufficient exposure to sun, use of sun protection, diet or supplements deficient in vitamin D, obesity, inborn or acquired genetic mutations in vitamin D metabolism, and deficient vitamin D-binding protein. Hypovitaminosis D leads to a variety of bone abnormalities such as osteoporosis, rickets, and osteomalacia. Vitamin D deficiency has also been associated with several diseases and conditions such as multiple sclerosis, cancer, cardiovascular disease, and aging processes (see Table 1 for list).[6-8] However, definitive evidence is lacking to link vitamin D with extraskeletal beneficial outcomes for cancer and cardiovascular disease.[9]

Table 1. Vitamin D deficiency is associated with several diseases and conditions.

Diseases and conditions associated with vitamin D deficiency	
Osteoporosis/osteomalacia/rickets	Depression
Asthma	Cancer
Heart health (high blood pressure, risk of cardiovascular disease)	Dementia/Alzheimer's disease in elderly
Inflammation	Peripheral artery disease
Rheumatoid arthritis	Chronic pain, bone pain
Lupus	Autism
Inflammatory bowel disease	Seasonal affective disorder
Types 1 and 2 diabetes	Anxiety
Increased cholesterol	Depression
Allergies	Cognitive impairment
Influenza and respiratory infection	Muscular sclerosis
Tuberculosis	Macular degeneration
Obesity	Aging
Overall mortality	Oral health
Athletic performance	Incontinence

Definition of Vitamin D Deficiency

Controversy has surrounded the definition of vitamin D deficiency, insufficiency, and sufficiency.[2,3,10]

The Endocrine Society's Practice Guidelines on Vitamin D define vitamin D deficiency as "a 25(OH)D <20 ng/mL, insufficiency as 21–29 ng/mL and sufficiency as at least 30 ng/mL for maximum musculoskeletal health.

This definition has also been accepted by the National Osteoporosis Foundation, International Osteoporosis Foundation, American Association for Clinical Endocrinologists, and the American Geriatric Society."[3,11]

The Institute of Medicine definition is based on minimal requirements for healthy bones and defines risk of vitamin D deficiency as 25(OH)D <30 nmol/L (12 ng/mL); risk of vitamin D inadequacy as 30–49 nmol/L (12–19 ng/mL); sufficiency as 50–125 nmol/L (20–50 ng/mL); and possible increased risk for harm when levels exceed 125 nmol/L (50 ng/mL).[1] In children and adults, excess vitamin D (hypervitaminosis D) is defined as concentrations of >250 nmol/L (100 ng/L) and intoxication as >375 nmol/L (150 ng/mL).[12-15]

Testing for Vitamin D

Both the Institute of Medicine and Endocrine Society agree that there is no need to screen populations that are not at risk of vitamin D deficiency.[10,11] Testing is recommended to confirm clinical symptoms of rickets in children and osteomalacia in adults, and also for those at risk, such as pregnant women, those with increased skin melanin pigmentation, children and adults who are obese, and those who abstain from direct sun exposure or with fat malabsorption syndromes, kidney disease, other risk factors, or inherited or acquired disorders in vitamin D metabolism.

How Is Vitamin D Tested?

Vitamin D testing has evolved over the years from methods that involved manual extraction of vitamin D using organic solvents, reconstitution of vitamin D, and measurement using immunoassay techniques.[4] This method had the advantage of producing accurate measurements due to thorough extraction of vitamin D from vitamin D-binding protein; however, the procedure is slow and labor-intensive.

Liquid chromatography mass spectroscopy (LC-MS/MS) is considered the most accurate method for determining the concentration of 25(OH)D; however, this method is laborious and requires specialized training for laboratory operators.

For higher-volume clinical laboratories, automated chemiluminescent assays have taken the place of the more-manual methods. Automated assays are less labor-intensive, higher-throughput, and meet the higher demand for increased testing; however, assay results from different manufacturers may not be the same (due to different antibodies, assay design, or other causes) and cannot be compared. In addition, automated assay results may be different from LC-MS/MS and manual methods.

Variability in Vitamin D Assays

The variability between 25(OH)D concentrations as measured by the various 25(OH)D assays and methods may come from several sources. Some assays have differential affinity for 25(OH)D_3 and 25(OH)D_2, which can cause inconsistencies in test results between assays, especially in cases of vitamin D_2 supplementation. Other causes of variability include incomplete extraction of vitamin D from its binding protein (e.g., in pregnant women, who have higher levels), cross-reactivity with the 24,25$(OH)_2$D metabolite, and matrix interferences.[2] Variability in results indicates the need for standardization of 25(OH)D assays.[2,16-21]

Standardization has been defined as a process whereby "all laboratories and assays are brought into alignment with the 'true concentration' based on gold standard reference measurement procedures and certified reference materials. That is, standardized laboratories report the 'true' concentration—in this case, of serum total 25(OH)D—regardless of time, place and assay or measurement system."[2]

CDC Vitamin D Standardization Program

In November 2010, the National Institutes of Health (NIH) Office of Dietary Supplements (ODS) established the vitamin D standardization program (VDSP), an international collaboration between the National Institute of Standards and Technology (NIST), the Centers for Disease Control and Prevention (CDC), and Ghent University (Ghent, Belgium).[16,18,22] The goals of the VDSP were to provide laboratories and manufacturers with reference materials (NIST) and a reference protocol (Ghent University) to standardize the laboratory measurement of vitamin D. The reference methods assign concentrations to reference materials. Reference materials calibrate all 25(OH)D assays and verify calibrations, such that assay results are traceable to a common standard and comparable between assays and laboratories (Figure 1, Table 2). Another VDSP goal was the standardization of 25(OH)D measurement in national health and nutrition surveys (including retrospectively) using reference materials and methods.[18,23,24]

Figure 1. Schematic of basic activities involved in standardization of assays performed at the National Institute of Standards and Technology (NIST), the Centers for Disease Control and Prevention (CDC), and individual laboratories. Standardization involves traceability to "true" gold-standard reference methods and materials in order to provide accurate results that can be compared between assays and laboratories and over time.

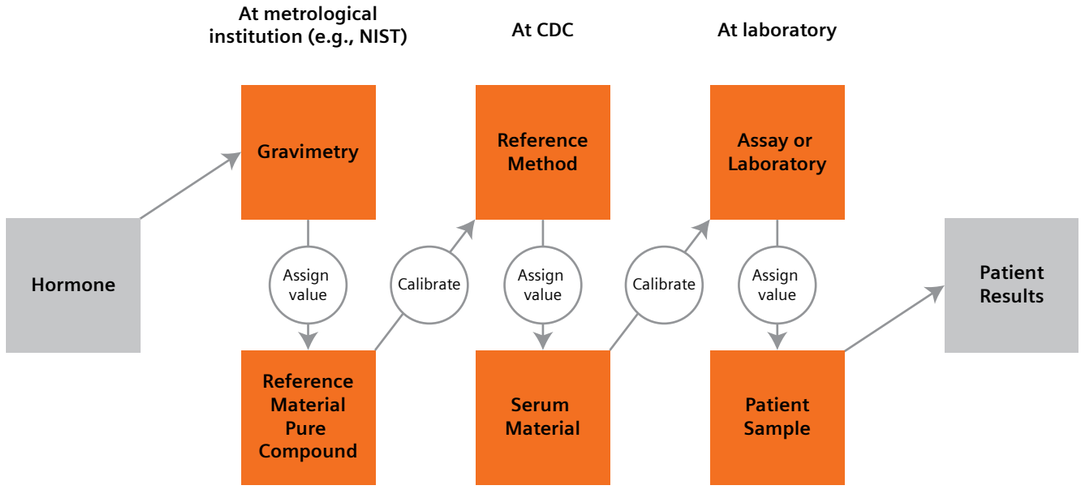


Table 2. Components of the CDC Hormone Standardization Program.

Reference Method at Reference Laboratory* • For total 25(OH)D (sum of 25[OH]D_2 and 25[OH]D_3) in serum uses HPLC-MS/MS. • Separately quantitates 25(OH)D_2 and 25(OH)D_3 with high level of specificity and is not affected by other vitamin D isomers, such as C3-epi-25(OH)D_3. • Meets stringent analytical performance criteria (maximum allowable bias: ≤1.7%; maximum allowable imprecision: ±5%).	Calibrated using "pure compounds" = primary reference material (e.g., SRM 2972a for total vitamin D from NIST). Thus, results are traceable to the International System of Units (SI) according to the International Organization for Standardization (ISO) standard for traceability in laboratory medicine. At CDC, primary reference material assigns values to blood samples = secondary reference materials using reference method procedures. Secondary reference materials calibrate assays.
Calibration in Laboratory with CDC Value-assigned Secondary Reference Material • For total 25(OH)D meets stringent analytical performance criteria (maximum allowable mean bias: ±5%; maximum allowable imprecision: <10%). • For total testosterone meets stringent analytical performance criterion (maximum allowable mean bias: ±6.4%)	VDSCP to monitor total vitamin D assay results over time with CDC-provided reference samples that are value assigned by the CDC. CDC HoST Certification Project to monitor total testosterone assay results over time using CDC-provided reference samples that are value-assigned by the CDC.
Laboratory Surveys • For total 25(OH)D meets stringent analytical performance criteria (maximum allowable mean bias: ±5%; maximum allowable imprecision: <10%). • For total testosterone meets stringent analytical performance criterion (maximum allowable mean bias: ±6.4%).	Collaboration with College of American Pathologists (CAP) and other proficiency-testing companies.
Training and Education	Instructions for end users of assays Procedures Reference ranges Monitor effectiveness of standardization

**https://www.cdc.gov/labstandards/vdscp_laboratory.html*

CDC Vitamin D Standardization Certification Program

To accompany the VDSP, the CDC established the Vitamin D Standardization-Certification Program (VDSCP) whereby manufacturers and laboratories may participate in an ongoing certification process (Figure 2).[25] The certification process involves a calibration stage followed by quarterly challenges of 10 samples per quarter, provided by the CDC and value-assigned by the Ghent University RMP. The CDC determines bias, precision, and total error according to Clinical and Laboratory Standards Institute (CLSI) Document EP9-A2. Certification is granted when the mean bias of the 40 Phase 2 samples is ±5% to the CDC and University of Ghent Vitamin D_2 and D_3 Reference Method Procedure, and overall imprecision is <10%. Certification must be renewed quarterly for the most recent four quarters. Several manufacturers of different 25(OH)D methods participate, and some have been certified since the inception of the VDSCP. To date, the Siemens Healthineers ADVIA Centaur® Vitamin D Total assay has achieved certification for seven consecutive years, the Atellica® IM Vitamin D Total Assay has achieved certification for two consecutive years, and the Dimension® EXL™ Vitamin D Total assay has achieved certification for three consecutive years.[26]

The international External Quality Assessment (EQA) scheme for vitamin D metabolites (DEQAS) was established in 1989 to monitor variability in 25(OH)D assays.[2,27]

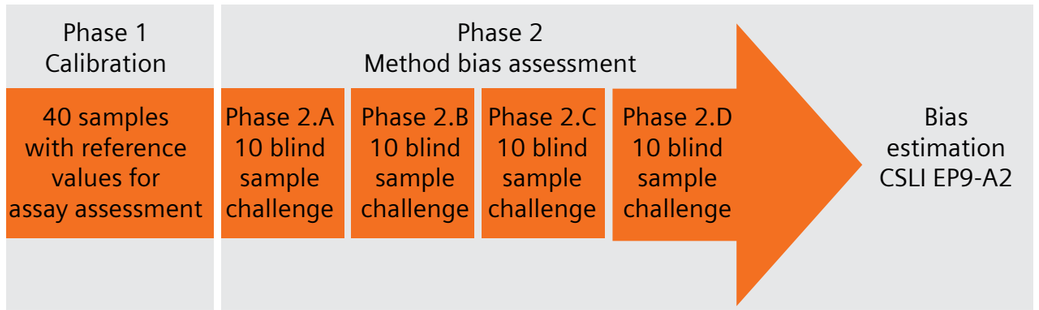
In 2013, the NIST Reference Method Procedure value-assigned target values for DEQAS materials, making DEQAS accuracy-based for 25(OH)D. This allowed for unbiased evaluation of assay variation. However, DEQAS results since 2013 still demonstrate considerable sample-to-sample variation within and between different assays and laboratories, underscoring the need for standardization of all assays.[2,22]

Another effort toward helping to reduce inconsistencies among assays was the development of a $24,25(OH)_2D_3$ Reference Method Procedure by NIST and its use in assigning values to SRMs 972a, 2973, and 2971, supported by the NIH ODS as part of the VDSP effort.[2,27]

Vitamin D Conclusion

The VDSP aligns the results of 25(OH)D assays to gold-standard reference methods developed by Ghent University and certified reference materials provided by NIST, thereby ensuring accurate results that can be compared among assays and institutions. For optimal patient care and outcomes, researchers, clinicians, and sponsors of national surveys should adhere to using the VDSP protocols for 25(OH)D methods and participate in ongoing certification by the CDC VDSCP. The use of Siemens Healthineers ADVIA Centaur, Atellica IM, and Dimension EXL Vitamin D Total assays that are standardized and CDC-certified should help ensure harmonization and accurate results and diagnoses, resulting in better patient care.

Figure 2. The CDC Vitamin D Standardization-Certification Program (VDSCP) consists of two phases providing 40 value-assigned reference samples for Phase I (calibration) and 10 blind sample challenges four times annually (total of 40 blind samples) for Phase 2.[25] The CDC then determines bias according to CLSI Document EP9-A2. Certification is awarded quarterly when results from the most recent four quarters have met the CDC criteria for precision (<10%) and mean bias (±5%).



What Is Testosterone?

Testosterone (4 androsten 17ß-ol-3-one) is a steroid hormone and the major androgen (male sex hormone) in males, which is produced by Leydig cells in the testes. Testosterone production is controlled by luteinizing hormone, which is released from the anterior pituitary acting directly on Leydig cells. In females, the major sources of testosterone are the ovaries, the adrenal glands, and the peripheral conversion of precursors, specifically the conversion of androstenedione to testosterone. Testosterone levels in women are about 10 times lower than in men.[28]

Abnormal Testosterone Levels

Disorders involving the male sex hormones (androgens) include primary and secondary hypogonadism, delayed or precocious puberty, and impotence in males, and hirsutism (excessive hair) and virilization (masculinization) due to tumors, polycystic ovaries, and adrenogenital syndromes in females.

Testing for Testosterone

Testosterone concentrations in the circulation are measured in the diagnosis and treatment of the disorders listed above (primary and secondary hypogonadism, delayed or precocious puberty, and impotence in males; in females, hirsutism and virilization due to tumors, polycystic ovaries, and adrenogenital syndromes). In recent years, increased demand for total testosterone testing has resulted from promising new therapies for diseases and conditions of testosterone excess or deficiency.

Testosterone strongly binds to plasma proteins such as sex hormone-binding globulin (SHBG) (65% of total testosterone) or testosterone-estradiol-binding globulin (TeBG). SHBG transports testosterone throughout the circulation and is a hormone reserve; testosterone bound to SHBG is biologically inactive. Testosterone also binds with low affinity to cortisol-binding globulins (CBG) and albumin. 30–40% of testosterone is bound to albumin, is easily removed, and considered biologically available. Less than 2.5% of total testosterone circulates unbound to plasma proteins (free), also considered biologically active. Total testosterone assays detect both bound and free testosterone concentrations in the blood. (It should be noted that in women, it is important to measure the amount of biologically available testosterone because SHBG concentrations are affected by a variety of factors, including thyroid and estrogen hormonal changes. High levels of active testosterone can be the cause of hyperandrogenemia in women who have total testosterone levels within the reference range.)

How Is Testosterone Tested?

Until the 1970s, extraction and radioimmunoassay (RIA) methods were used to measure testosterone. RIA methods can yield higher accuracy than immunoassays in current use; however, time and cost are major disadvantages for routine use of RIA methods. In the late 1970s, extraction RIA methods were replaced with direct RIAs that did not require extraction or chromatography.

Subsequently, direct immunoanalytical methods with nonradioactive markers were developed for use on analyzers. Direct immunoassays are easy to use and more convenient for routine clinical practice but lack adequate specificity, have higher values than classical RIA, and incompletely extract testosterone from binding proteins, particularly SHBG, which results in less of the total analyte for measurement. This is a problem when measuring very low concentrations, such as in women. Currently in Europe, most laboratories use immunoassays and no extraction; results are obtained quickly, but accuracy is low.[29]

LC-MS/MS was introduced in the 1990s and 2000s and is now considered the gold-standard method. LCMS/MS demonstrates the highest accuracy at low concentrations and is useful in women. However, this method is often prohibitive due to cost, the need for trained personnel and standardization and validation by each laboratory, and interference by conjugates. LC-MS/MS also has challenges associated with commercial kits whose results are not always more accurate than those of immunoassays.

Variability in testosterone assays

Significant variability in measurements is observed when comparing results from various testosterone assays, particularly at low concentrations, such as found in hypogonadal males, children, and women. Variability relates to measurement inaccuracy and lack of specificity, sensitivity, and precision/repeatability. As in the case of total vitamin D, the clinical and research communities have called for the standardization of testosterone testing. Standardization of testosterone testing using the CDC reference method and materials has been proposed to address variability issues with respect to reference ranges of different groups such as women, men, age, and phase of menstrual cycle.[30] Indeed, a recent publication has demonstrated the feasibility of harmonizing reference ranges in men across assays that generate variable results by calibrating to the CDC reference method and materials.[31]

CDC Standardization of Testosterone Assays

In 2007, the Endocrine Society recommended "accuracy-based testing of testosterone and calibration of all methods traceable to a single high-level reference material."[30,32-34] Standardization of testosterone assays aims to help ensure accurate and comparable results across testing systems (assays), laboratories, and time, thereby improving quality of patient care, clinical research, and epidemiological studies, including the development of evidence-based guidelines. Similar to the VDSP, the HoST for testosterone goals were to develop "true-value" reference materials and reference methods. Reference methods assign values to reference materials. Reference materials calibrate assays and verify calibrations so that different testing facilities and assays can trace their results to a common standard (Figure 1). Using nonstandardized tests increases the chance of misdiagnosis and wrong treatment and the inconvenience and increased costs caused by retesting.

In 2010, The Endocrine Society and eleven other organizations made the following recommendations toward improving testosterone measurements:[30] First, all users and stakeholders of testosterone assays in the public and private sectors should support the CDC testosterone standardization procedures and demand that manufacturers and laboratories develop accurate and reliable tests worthy of research funding and third-party payer reimbursement. Second, experts should provide total testosterone performance criteria over the full range of values for children, adults, and each sex using standardized methods. Third, reference range values should be determined using standardized methods for children, adults, and each sex. Fourth, experts should provide guidelines for consistent sample collection and preparation for standardized assays. Fifth, third-party payers and health care organizations should support the use of assays that have been standardized. Sixth, funding bodies and journals should only support and consider for publication research performed with standardized assays demonstrating accuracy. Tests selected for patient care, research, and public health activities should be standardized. New testosterone tests should be standardized to the CDC. Seventh, manufacturers and laboratories should continue to develop new methodological approaches for accurate measurement of testosterone; emphasis should be placed on results, not methodology. Standardized testosterone testing should yield comparable test results across methods and time. Currently, similar to the VDSCP, on a quarterly basis, the CDC grants certification to those assays that pass acceptance limits in the CDC HoST Certification Program over the most recent four quarters. The HoST acceptance criterion is ±6.4% mean bias to the CDC Testosterone Reference Method.[35] To date, the Siemens Healthineers ADVIA Centaur Testosterone II assay has achieved certification for two years and the Dimension EXL Serum Total Testosterone assay has achieved certification for four consecutive years.[26] Between 2012–2013 and 2016, CDC-directed accuracy-based proficiency testing demonstrated that about 15% more participating laboratories had improved analytical accuracy and precision; however, improvements are still needed, especially at lower concentrations.[35,36]

Testosterone Conclusion

The CDC HoST program for total testosterone assays provides reference methods and materials that help ensure sensitive and reliable detection of accurate total testosterone concentrations. Standardization of total testosterone assays allows a comparison of results across different assays, national surveys, and over time. Consensus documents prepared by experts recommend that all publications and national surveys use total testosterone assays that are standardized using the CDC HoST Program. Use of the ADVIA Centaur Testosterone II and Dimension EXL Total Testosterone assays that are standardized and CDC-certified should help ensure harmonization, accurate results and diagnoses, and improved patient care.

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