

Laboratory Procedure Manual

Analyte: **25-Hydroxyvitamin D**

Matrix: **Serum**

Method: **Diasorin (formerly Incstar) 25-OH-D assay**

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as performed by:

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Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

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Public Release Data Set Information

This document details the Lab Protocol for NHANES 2001-2002 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
L06VID_b	LBDVIDMS	Vitamin D LCMSMS (nmol/L)

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1. Summary of Test Principle, Clinical Relevance, and Analytical Note for 25-Hydroxyvitamin D Data Analysis using NHANES III (1988-1994), NHANES 2001-2006, and NHANES 2007-2010

Vitamin D is functionally a hormone, rather than a vitamin, and is one of the most important biological regulators of calcium metabolism, in conjunction with parathyroid hormone and calcitonin. As calciferol enters the circulation, it is metabolized to several forms, the primary one being 25-hydroxycalciferol (25-OH-D) (1). The first step in the metabolism of vitamin D, 25 hydroxylation, occurs mainly in the liver (2). In humans, only a small amount of 25-OH-D is metabolized in the kidney to other di-hydroxy metabolites (3, 4). Because 25-OH-D is the predominant circulating form of vitamin D in the normal population, it is considered to be the most reliable index of people's vitamin D status (5). Vitamin D₃ (cholecalciferol) is the naturally occurring form of vitamin D produced in the skin after 7-dehydrocholesterol is exposed to solar UV radiation. Vitamin D₂ (ergocalciferol) is produced synthetically by UV irradiation of ergosterol. The two forms differ in the structures of their side chains, but they are metabolized identically and have equivalent biological activities. Both forms are used for fortification of dairy products. Because these two parent compounds provide various contributions to people's overall vitamin D levels, it is important that both forms are measured equally (5, 6).

The measurement of 25-OH-D (referred to as the vitamin D assay) is becoming increasingly important in the management of patients with various disorders of calcium metabolism associated with rickets, neonatal hypocalcemia, pregnancy, nutritional and renal osteodystrophy, hypoparathyroidism, and postmenopausal osteoporosis (7-11).

The Diasorin (formerly Incstar) 25-OH-D assay consists of a two-step procedure. The first procedure involves a rapid extraction of 25-OH-D and other hydroxylated metabolites from serum or plasma with acetonitrile. Following extraction, the treated sample is assayed by using an equilibrium RIA procedure. The RIA method is based on an antibody with specificity to 25-OH-D. The sample, antibody, and tracer are incubated for 90 min at 20-25 °C. Phase separation is accomplished after a 20-minute incubation at 20-25 °C with a second antibody-precipitating complex. An NSB/Addition buffer is added after this incubation prior to centrifugation to aid in reducing non-specific binding.

See Analytical Note for 25-Hydroxyvitamin D Data Analysis using NHANES III (1988-1994), NHANES 2001-2006, and NHANES 2007-2010
at <http://wwwn.cdc.gov/nchs/nhanes/VitaminD/AnalyticalNote.aspx>

2. Safety Precautions

The vitamin D assay employs ¹²⁵I as a tracer (at a level that does not exceed 4 µCi). Therefore, observe all necessary radiation safety considerations for isotope management and disposal according to the guidelines of the CDC *Radiation Safety Manual*. In addition, all personnel must successfully complete the CDC training course *Radiation Safety in the Laboratory*, or demonstrate knowledge equivalent to those who did. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus, observe Universal Precautions; wear protective gloves during all steps of

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this method because of both infectious and radioactive contamination hazards. (We recommend the hepatitis B vaccine series for all analysts working with intact blood and serum sample materials.) Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines

Two reagents of special concern in this kit are sodium azide and acetonitrile. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. When disposing of this reagent, flush it with a large volume of water to prevent azide build-up (12).

Acetonitrile is a flammable substance, and exposure to its liquid or vapor is harmful. If exposure occurs, remove contaminated clothing, flush affected areas with copious amounts of water, and call a physician. If someone inhales acetonitrile, move him or her to fresh air, and give artificial respiration and oxygen if respiration is impaired. In case of a fire, extinguish it with dry chemicals or carbon dioxide. In case of a spill, carefully remove and dispose of the acetonitrile according to environmental regulations.

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDSs) for these chemicals are readily accessible as hard copies in the lab. If needed, MSDS for other chemicals can be viewed at <http://www.ilpi.com/msds/index.html> or at <http://intranet.cdc.gov/ohs>.

3. Computerization; Data System Management

- A. Statistical evaluation and calculation of the run are accomplished with the software on the Titertek Apex 10/600 Plus gamma counter and Packard COBRA gamma counter. After a run is complete and any additional corrections by the analyst are made, the result file (containing the patient data as well as the QC data) is electronically transferred to the appropriate analyte-specific subfolder in Q: /ITN/Nutrition Lab/Import into Access on the NCEH/DLS Local Area Network (LAN). The analyst also gives a hardcopy of the result file to the reviewing supervisor. After the reviewing supervisor approves the final values for release by checking off the bench and blind QC values and signing the hardcopy, he/she sends an email to the computer support staff that the data has been released to be imported into the NHANES 1999+ database that is located in Microsoft Access; the computer support staff imports the data into the NHANES 1999+ database by using a macro. Data entry is verified by the computer support staff and the supervisor. Data is transmitted electronically several times weekly to Westat's ISIS computer system, and transferred from there to NCHS. Abnormal values are confirmed, and codes for missing data are entered by the analyst and are transmitted as part of the data file to the Westat ISIS computer, and are eventually forwarded to NCHS. Westat also prepares the abnormal report notifications for the NCHS Survey Physician.
- B. Files stored on the network or CDC mainframe are automatically backed up nightly by DLS LAN support staff and CDC Data Center staff, respectively. Backup of the daily data containing all raw data files and result files for each run are the responsibility of the analyst. Typically these files are backed up once a week onto a floppy disk or a CD-

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ROM using a CD writer.

- C. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

- A. Although a fasting specimen is recommended, it is not required. No special instructions such as special diets are required. Diurnal variation is not a major consideration.
- B. Specimens for vitamin D analysis should be fresh or frozen serum. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers. Serum specimens should be stored at $\leq -20^{\circ}\text{C}$
- C. A sample volume of 50 μL is required for the assay; 150 μL will permit repeat analysis and adequate pipetting volume as well.
- D. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.
- E. Because vitamin D is very stable, serum samples may be frozen at -20°C to -70°C for years before analysis. Several freeze-thaw cycles do not seem to adversely affect the assay, although repeated freeze-thaw cycles should be avoided.
- F. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site of collection.
- G. Moderately hemolyzed specimens may be used because red blood cells do not contain vitamin D.
- H. Specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Nutritional Laboratory and the electronic copy of this file is located at Q: /ITN/Nutrition Laboratory/CLIA). The protocol discusses collection and transport of specimens and the special equipment required. In general, serum should be transported and stored at no more than -20°C . Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood or plasma should be transferred into a sterile Nalge cryovial labelled with the participant's ID.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this procedure

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6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

A. Reagent Preparation

(1) Donkey-anti-goat (DAG) Precipitating Complex

Donkey anti-goat serum, normal goat serum, and polyethylene glycol are diluted in a bovine serum albumin-borate buffer containing antimicrobial reagents. Although no reconstitution is necessary, mix the reagent for 5-10 min before and during use to ensure that a homogeneous suspension is achieved. If the reagent is not entirely used in one analytical run, store it at 2-8 °C until the expiration date on the label.

(2) Standards, quality control materials, and reagents

Standards, quality control materials, and reagents are supplied ready to use, with no reconstitution necessary. If not used in one run, the kit contents should be stored at 2-8 °C until the expiration date on the label. The 25-OH-D₃ tracer contains ¹²⁵I and should be properly handled with gloves and disposed of according to CDC radiation safety guidelines.

B. Standards Preparation

Vitamin D Standards (0.0, "A"/5.0, "B"/12.0, "C"/20.0, "D"/40.0, and "E"/100.0 ng/mL) are supplied as prediluted 25-OH-D₃ in processed human serum in a liquid form, ready to be used. If the entire kit is not used in one run, store the standards at 2-8 °C for up to 10 days. These standards are prepared by Diasorin (formerly Incstar) and are calibrated independently (13) against HPLC-purified 25-OH D.

C. Preparation of Quality Control Materials

Five levels of bench quality control materials are used. Normal and elevated levels of 25-OH-D in serum controls (the later produced by spiking with purified 25-OH-D standard solution) are provided with the kit. Three additional levels of serum pools have been prepared by CDC to complement the kit QC materials, in an effort to use in-vivo 25-OH-D concentrations. At present, few other sources of commercial QC materials are available with defined Diasorin (formerly Incstar) target levels, although the Bio-Rad Laboratories ECS Division (Anaheim, CA) is attempting to develop such materials.

Two levels of blind QC pools may be prepared from pooled, filter-sterilized fasting human serum obtained from donors with elevated or decreased vitamin D levels. Target levels are about 15 ng/mL for those with decreased levels and 50 ng/mL for those with elevated levels. Pool the serum in acid-cleaned 20-L glass carboys and mix well on a magnetic stirrer. Clean-filter the serum through stacked Millipore filters in a sequential manner using the filters of the following pore sizes, each preceded by a prefilter: 3.00-µm, 1.20- µm, 0.80- µm, 0.65- µm, 0.45- µm, 0.30- µm and 0.22- µm. Using sterile technique under a laminar-flow hood and a Micromedic Digiflex, dispense the serum in 500-µL aliquots into 2.0-mL Nalge cryovials. Cap and label the vials with

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NHANES bar-coded labels that have been specially prepared for the QC pools. Store the pools at $\leq -70^{\circ}\text{C}$ at the CDC Serum Bank in Lawrenceville where they will be

inserted randomly into the NHANES runs. Select twenty vials of each level at random for characterization of the quality control limits and for testing of homogeneity.

D. Other Materials

- (1) Diasorin (formerly Incstar) Corporation 25-Hydroxyvitamin D ^{125}I RIA kit, 100-test size (cat. no. 68100E, Diasorin (formerly Incstar) Corporation, Stillwater, MN).
- (2) Disposable 12- x 75-mm borosilicate glass tubes (American Scientific Products, McGaw Park, IL).
- (3) Three levels of normal human serum quality control pools prepared in-house at CDC to complement two levels of QC pools provided with the kit. Materials are dispensed as 250- μL aliquots in tightly capped Nalge cryovials and stored at -70°C until used.
- (4) Corks, size 2 (any vendor).
- (5) Parafilm, 4 inch rolls (any vendor).

E. Instrumentation

- (1) Packard COBRA Quantum Gamma Counter (Packard Instrument Company, Downers Grove, IL).
Or: Titertek Model Apex 10/600 Plus Gamma Counter (Titertek Instruments, Huntsville, AL).
 - (2) Beckman J6-HC centrifuge, 222-tube capacity, temperature-controlled (Beckman Instruments, Inc., Palo Alto, CA).
Or: Jouan CR412 centrifuge (Jouan, Inc., Winchester, Virginia).
- NOTE:** Centrifuge should be capable of $1800 \times g$.
($g = 1118 \times 10^{-8}$) (radius in cm) (rpm) 2 .
- (3) Gilson Pipetman pipettes, 25- and 50- μL sizes (Rainin Instrument Co., Woburn, MA).
 - (4) Eppendorf Repeater 4780, 1.0- to 5.0-mL size (Brinkmann Instruments, Westbury, NY), to deliver 50-, 500-, and 1000- μL volumes.
 - (5) Packard Multiprobe II Liquid Handling System (Packard Instrument Company, Downers Grove, IL) IBM compatible computer, and 96-tube sample racks (Hook and Tucker Zenyx Corporation, Manchester, U.K.)
 - (6) Vortex mixer (Fisher Scientific, Fairlawn, NJ).

7. Calibration and Calibration Verification Procedures

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Diasorin (formerly Incstar) prepares Vitamin D calibration standards from HPLC-purified 25-OH-D₃ and reverifies them in its reference laboratory using a reference HPLC assay (13). These standards -- 5, 12, 20, 40, and 100 ng/mL -- are run with each assay. At present, the only available National Institutes of Standards and Technology Standard Reference Material (SRM) with target values assigned for vitamin D is a coconut-milk matrix material, which is not suitable for this assay.

A. Performance checks for the assay include:

- Nonspecific binding: The zero standard is used as the nonspecific binding. The CPM for the NSB tubes should be <5% of the CPM of the total counts.
- Maximum binding: The CPM of zero ng/mL standard should be >28% of the CPM of the total counts.
- Slope of the standard curve: The 80% and 50% points of the standard curve should be monitored for run-to-run reproducibility.

In accordance with the NCCLS method comparison protocol, a 40-sample comparison between the reference HPLC method (either the fully-validated in-house CDC HPLC method or the HPLC method of Dr. Bruce Hollis, Medical University of South Carolina - developer of the kit) may be performed in order to establish comparability.

B. Calibration of Instrument

The Packard Cobra gamma counter is used for data reduction. To ensure the accuracy of test results, operators must do the following:

- Daily: Background and efficiency are run simultaneously using Polymedco Isocalibrators and associated Packard software. Efficiency should be at least 75% for ¹²⁵I. Printout indicates if all performance parameters are within acceptable limits.
- Monthly: Perform normalization with Isocalibrators according to procedure outlined in Packard Procedure manual. Printout will indicate if performance parameters are within acceptable limits.
- Semiannually: Preventative maintenance through Packard Technical Service Inspection.

C. Instructions for Daily Calibration of Instrument

- (1) Load ¹²⁵I Isocalibrators in positions 2, 6, 10, 14 and 18, with blank tubes in positions 1, 5,9,13, and17 in rack labeled with protocol clip 25.
- (2) Select F2 (SC Commands), F6 (next protocol)

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A. Quick Reference Summary Table (Table 1, APPENDIX)

B. Extraction Procedure

- (1) Using the Multiprobe II, open the file VitD ACN.MTH. Load standards, controls, and samples into the sample rack and place the rack on the Multiprobe II as indicated.
- (2) Label a 12- x 75-mm disposable glass tube for each standard, control and patient sample and place the tubes into the recipient rack.
- (3) Add 500 μ L of acetonitrile to each recipient tube using the Eppendorf Repeater pipet. Place the rack on the Multiprobe II as indicated.
- (4) Select "Execute Test" and then select "Next" in the Initial User Query Test Startup Page box. In the Query Page 1 box, enter the number of samples +10 and select "Start" to transfer 50 μ L of the standard, control, or unknown serum specimen to a recipient tube. Make sure that the samples are dispensed slowly and that the pipet tips are below the surface of the acetonitrile while dispensing.
- (5) After each sample is delivered, place a cork in the top of the tube and vortex the contents for 10 sec.
- (6) Centrifuge the samples using 1200 x g for 10 min at 20-25 $^{\circ}$ C.
- (7) Using the Multiprobe II, open the file Vit D Assay MTH. Place the rack of centrifuged samples and the recipient racks containing duplicate labeled 12- x 75-mm glass reaction tubes on the Multiprobe II as indicated. To pipet duplicate 25 μ L aliquots from the supernatant into the recipient tubes, select "Execute Test" and then select "Next" in the Initial User Query Test Startup Page box. In the Query Page 1 box, enter the number of samples + 10, then select "Start". (Do not remove the corks until immediately prior to sample transfer.)
- (8) **CAUTION:** Take care not to disturb the pellet.
- (9) Proceed with the assay procedure.

C. Assay Procedure

- (1) Allow all reagents to equilibrate to ambient room temperature. Do not allow any reagents to warm above 25 $^{\circ}$ C.
- (2) Using the Eppendorf Repeater pipet, add reagents to the reaction tubes as follows:
 - (a) Total Counts Tubes

50 μ L of 125 I 25-OH-D
1.0 mL of NSB buffer

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b) Nonspecific Binding Tubes

25 µL of 0.0 standard (extracted)
50 µL of ¹²⁵I 25-OH-D
1.0 mL of NSB buffer

(c) Standards, Controls and Unknown Samples

25 µL of standard, control, or unknown sample (extracted)
50 µL of ¹²⁵I 25-OH-D
1.0 mL of 25-OH-D antiserum

- (3) Cover the tops of the tubes with a sheet of Parafilm and vortex them gently without allowing the contents to foam. Incubate tubes for 90 (+/- 10) minutes at 20-25 °C.
- (4) Using the repeating dispenser, add 500 µL of DAG precipitating complex (DAG precipitating complex should be mixed thoroughly before and during use) to all tubes except the total counts tubes.
- (5) Mix tubes well and incubate the tubes for 20-25 minutes at 20-25 °C.
- (6) Add 500 µL of NSB/Addition buffer to all tubes except the total count tubes. Cover and vortex gently to mix tubes well. Use caution to avoid splashing due to high liquid level in tubes.
- (7) Centrifuge all tubes except total counts for 20 minutes at 20-25 °C using 1800 x g.
- (8) Decant the supernatants (except the total counts tubes).
- (9) Using the gamma counter, count each tube for a minimum of 1 minute to achieve statistical accuracy.

D. Calculation

Both the Packard COBRA and Titertek Apex 10/600 Plus counters have full data reduction capabilities. Linear B/B₀ vs log₁₀ concentration with a cubic or auto spline curve fit is used in both counters where:

$$B/B_0 = \frac{\text{CPM of Standard or Unknown Sample} - \text{CPM of NSB}}{\text{CPM of 0 Standard} - \text{CPM of NSB}} \times 100$$

and B = corrected counts/min (blank subtracted) for each tube, and B₀ = corrected counts/min of 0 standard (blank subtracted).

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This method results in a linearized 5-point standard curve with an inverse relationship of levels of radioactivity (measured in counts per min, or CPM) to concentration of vitamin D in the serum sample. Serum results are expressed as nanograms of vitamin D per milliliter of serum (ng/mL).

E. Special Procedure Notes – CDC Modifications

This method was brought on line in January 1994 as a service method in the NHANES Laboratory, and no modifications of the manufacturer's instructions were made.

9. Reportable Range of Results

The method described here is designed to detect serum 25-OH-D values from 5 to 100 ng/mL, which is beyond the normal range of values expected to be observed for human serum 25-OH-D concentration. Values <5.0 and >70 ng/mL are verified by re-assay, including re-extraction. For re-extraction, dilute elevated specimens (>100 ng/mL) with 0 standard prior to extraction. Any samples with CVs >10% are also re-assayed.

The sensitivity of this assay, when defined as the lowest quantity differentiated from zero at 2 standard deviations below the mean cpm of the zero standard, has been shown to be at or below 1.5 ng/mL. Any values less than the lowest standard, 5.0 ng/mL, are reported as "3.0." These levels could occur physiologically and would indicate severe 25-OH-D deficiency. Values greater than 70 ng/mL indicate prolonged exposure to ultraviolet radiation or excessive supplementation.

10. Quality Control (QC) Procedures

The two levels of 25-OH-D QC materials included with each kit are prepared by the manufacturer from human serum spiked with appropriate amounts of 25-OH-D₃ to obtain concentrations within specified ranges. Sodium azide is added as an antimicrobial. These pools are analyzed in duplicate at the beginning of each run.

In every batch of 20 specimens analyzed, either one low- or one high-concentration blind quality control pool is randomly inserted. The blind pools are aliquoted and labeled in exactly the same fashion as the NHANES 1999+ specimens and are inserted in the specimen batches by the Serum Bank personnel when the specimens are received from the field and racked for analysis.

Like blind QC pools, bench QC pools may also be made from filter-sterilized fasting human serum, as described in Section 6.c. An effort is made to obtain serum from individuals who are outdoors every day, such as joggers, and from persons who greatly restrict their outdoor activities. In this manner, one may obtain low-normal, intermediate and high in-vivo concentrations of QC pools in a matrix identical to that of unknown specimens. "High" concentration pools are prepared by spiking normal sera with purified 25-OH-vitamin D (Sigma) to desired levels (e.g., > 50 ng/mL). These pools are prepared in the same manner as patient samples and analyzed in duplicate at the beginning and end of each run.

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The results from the pools are checked after each run. The system is declared "in control" if all three QC results are within 2s limits and the run is accepted. If one of the three QC results is outside the 2s limits then apply rules below and reject if any condition is met - the run is then declared "out of control":

- 1_{3s} Any of the three QC results are outside the 3s limit
- 2_{2s} Two of the three QC results in the run are outside the 2s limit (same side of mean)
- R_{4s} Sequential QC results (either within the run or across runs) are outside the 2s limit on the opposite sides of the mean
- 10_x Ten sequential QC results (across pools and across runs) are on the same side of the mean

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared "out of control" for that analyte as assessed by internal (bench) QC. The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated quarterly. When necessary, limits are updated to include more runs.

While a study is in progress, electronic copies of the QC results from each run are stored in the analyte-specific folder on Q: /ITN/Nutrition Lab/Data handling/Import into Access. Electronic copies of the tracking of the QC results over time are stored in the analyte-specific folder on Q: /ITN/Nutrition Lab/Data handling/QC Results in Excel. A hardcopy of the QC results from each run is also kept by the analyst.

11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

If the system should be declared "out of control," repeat the entire run. If the "out of control" condition still exists, use a new kit and evaluate the autodiluter for pipetting precision and accuracy. Reassay specimens for that analytical run after the system has been reverified to be "in control."

12. Limitations of Method; Interfering Substances and Conditions

The greatest source of imprecision in this method is likely to be the extraction step. Other sources of procedural imprecision may be intermittently imprecise micropipettors or outdated reagents. If the initial concentration of a specimen is greater than that of the highest standard, the specimen should be diluted with 0 standard prior to re-extraction. Counting times should be sufficient to prevent statistical error (e.g., the accumulation of 2,000 CPM will yield 5% error, while the accumulation of 10,000 CPM will yield 1% error). Specimens should not be repeatedly frozen and thawed.

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The kit antibody will demonstrate 2.5-100% cross-reactivity with all forms of di-hydroxy-vitamin D₂ and D₃ steroids.

13. Reference Ranges (Normal Values)

Season, race, and dietary intake are all known to affect the normal levels of 25-OH-D. 25-OH-D levels correlate well with ultraviolet radiation exposure. The reported difference in 25-OH-D values attributable to seasonal variation in ultraviolet radiation illustrates the importance of personal exposure to sunlight (14,15). The highest levels of 25-OH-D are found during the summer months, and the lowest levels during the winter. Race has also been shown to significantly influence the normal levels of 25-OH-D. It has also been reported that the mean plasma level of 25-OH-D in whites is greater than that in blacks (16).

Published normal ranges from smaller studies in the United States indicate an expected range of approximately 10-40 ng/mL. The NHANES 1999+ data will be used to define the normal U.S. levels when statistical analyses of these data are complete. Preliminary unweighted data from NHANES III suggest a normal range of 10-55 ng/mL.

14. Critical Call Results (“Panic Values”)

Because we are collecting data for national prevalence purposes only, we do not routinely notify survey participants with abnormal 25-OH-D values.

15. Specimen Storage and Handling during Testing

Specimens should remain at room temperature during preparation and testing, and then be promptly refrozen for storage.

16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

There are no acceptable alternative methods for performing this test for NHANES 1999+. In case of system failure, store all specimens at ≤ -20 °C until the system is functioning.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Test results are reported to the collaborating agency at a frequency and by a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file or Excel file, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically several times weekly to the Westat ISIS computer and then are transferred to NCHS. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

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18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

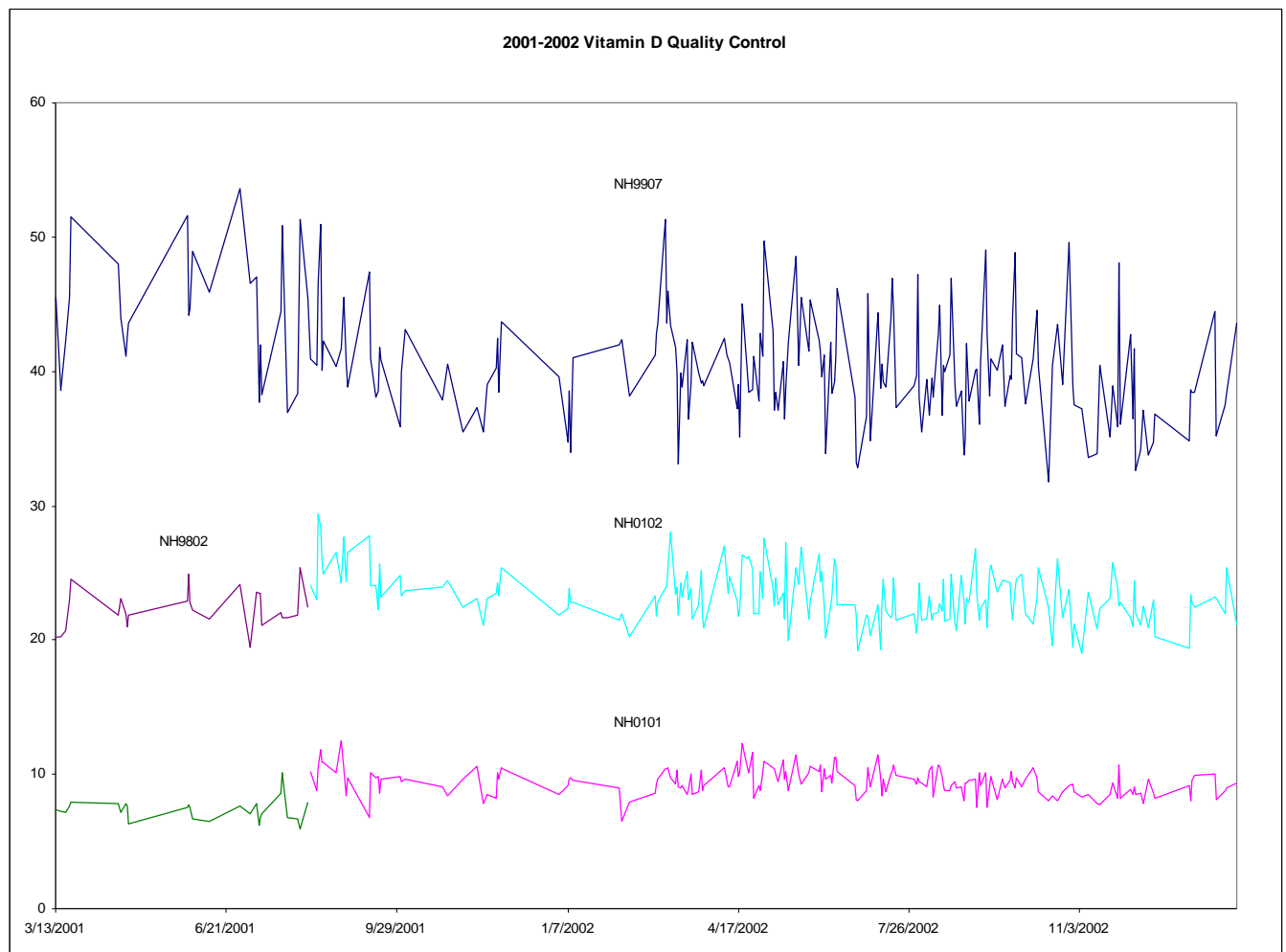
The Microsoft Access database is used to keep records and track specimens for NHANES 1999+. If serum 25-OH-D analyses are used for smaller, non-NHANES studies, records are kept on files in Q:\ITN\Nutrition Lab on the DLS LAN.

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19. Summary statistics and qc graphs

Summary Statistics for Vitamin D by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
NH9801	27	3/13/2001	8/8/2001	7.348	0.827	11.3
NH9802	27	3/13/2001	8/8/2001	22.334	1.455	6.5
NH9907	218	3/13/2001	2/3/2003	40.697	4.174	10.3
NH0101	191	8/9/2001	2/3/2003	9.443	0.981	10.4
NH0102	191	8/9/2001	2/3/2003	23.238	1.934	8.3



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Appendix (Tables 1-3)

Table 1
Diasorin (formerly Incstar) 25-Hydroxyvitamin D Flow Table

	Total Cts	NSB	Standards* (ng/mL)						Control and Unknown Samples*			
			0	A/5	B/12	C/20	D/40	E/100	1	2	3	4
Tube number	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
Extracted 0 Standard*		25 µL	25 µL									
Extracted Standards*				25 µL	25 µL	25 µL	25 µL	25 µL				
Extracted control & Unknown Samples									25 µL	25 µL	25 µL	25 µL
¹²⁵ I 25-OH-D	50 µL											
NSB buffer	1 mL	1 mL										
25-OH-D antiserum			1.0 mL									
	Mix well, incubate for 90 min (± 10 min) at 20-25 EC.											
DAG Precipitating Complex			500 µL									
	Mix well; incubate for 20-25 min at 20-25 °C.											
NSB/ Addition Buffer			500 µL									
	Centrifuge using 1800 x g for 20 min.											
	Decant the supernatants.											
	Count each tube in gamma counter for 60 sec or longer.											

* Standards and control and unknown samples must be extracted with acetonitrile prior to the assay.

Table 2
NHANES 1999+ Serum Vitamin D Quality Control Pools

Pool	Mean (ng/mL)	N	Total Std. Dev.	Total CV (%)	2SD Limits	3SD Limits
0101	9.764	163	1.124	11.512	7.516-12.012	6.392-13.136
0102	24.113	163	2.411	9.997	19.292-28.935	16.881-31.345
9907	40.657	163	4.122	10.139	32.413-48.901	28.291-53.024

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Table 3

Historical Perspective: QC Pools Used Since The Conclusion of the NHANES III Vitamin D Analyses in 1995

Pool	Mean (ng/mL)	N	Total Std. Dev.	Total CV (%)	95% Limits	99% Limits
9501	6.88	37	2.34	33.93	2.60-11.17	1.24-12.52
9502	35.9	36	4.89	13.61	26.82-45.02	23.94-47.90
9601	8.3	38	1.83	22.09	6.11-10.48	5.42-11.17
9602	22.1	38	3.20	14.49	16.24-27.89	14.40-29.74
9603	32.3	38	4.70	14.54	25.82-38.83	23.76-40.89
9801	7.62	19	1.47	19.36	5.77-9.47	5.18-10.05
9802	22.97	24	3.21	13.97	18.07-27.87	16.51-29.42