Laboratory Procedure Manual

Analyte: **25-Hydroxyvitamin D**

Matrix: **Serum**

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

Method No:

Revised: **October 2015**

As performed by:

Nutritional Biochemistry Branch Division of Laboratory Sciences National Center for Environmental Health

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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.

Public Release Data Set Information

This document details the Lab Protocol for NHANES III (1988-1994) data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
l06vid_NH3	LBDVIDMS	Vitamin D LCMSMS (nmol/L)

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 D_3 (cholecalciferol) is the naturally occurring form of vitamin D produced in the skin after 7-dehydrocholesterol is exposed to solar UV radiation. Vitamin D_2 (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 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.

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 ^{125}I 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 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.

Material safety data sheets (MSDSs) for all chemicals contained in the kit are available in the MSDS section of the "Working Safely With Hazardous Chemicals" notebook, which is located in the laboratory as well as on the EHLS LAN.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Statistical evaluation and calculation of the run are accomplished with the ISODATA software on the Micromedic Apex gamma counter.

   After the data are calculated and the final values are approved by the reviewing supervisor for release, the results are transcribed by the data entry clerk into the NHANES III data base that is located in RBASE on the NCEH/EHLS PC network; data entry is verified by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the values to the NCHS mainframe computer along with the other NHANES III data.

b. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

c. Documentation for data system maintenance is contained in hard copies of data records.

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 ≤-20 °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 effect 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 Division protocol for whole blood collection and handling (copies available in the NHANES laboratory and Special Activities Branch specimen handling office). The protocol discusses collection and transport of specimens and the special equipment required. In general, whole blood specimens should be transported and stored at no more than 4 °C. Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen at <-20 °C. Samples thawed and refrozen several times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, transfer the appropriate amount of serum into a sterile Nalge cryovial labeled with the participant's ID.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Micromedic Apex Automatic Gamma Counter (Micromedic Division, ICN Biomedical, Costa Mesa, CA).

Or: Micromedic Model 10/600 Plus Gamma Counter (ICN Biomedical).

(2) Beckman J6-B centrifuge, 222-tube capacity, temperature-controlled (Beckman Instruments, Inc., Palo Alto, CA).

Or: Beckman TJ-6 centrifuge (Beckman Instruments).

NOTE: Centrifuge should be capable of 1800 x g.

$(g = 1118 \times 10^{-8})(\text{radius in cm})(\text{rpm})^2$.

(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) Quatro 240 Sample Processor with Quatro Concerto software, IBM compatible computer, and 96 tube sample racks (Matrix Technologies Corporation, Lowell, MA).

(6) Vortex mixer (Fisher Scientific, Fairlawn, NJ).

b. **Materials**

(1) INCSTAR Corporation 25-Hydroxyvitamin D ^{125}I RIA kit, 100-test size (cat. no. 68100, 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 200-µL aliquots in tightly capped Nalge cryovials and stored at $\leq$-70 °C until used.

(4) Corks, size 2 (any vendor).

(5) Parafilm, 4 inch roll (any vendor).

c. **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 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-D_3 tracer contains ^{125}I and should be properly handled with gloves and disposed of according to CDC radiation safety guidelines.

d. **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-D_3 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 INCSTAR and are calibrated independently (13) against HPLC-purified 25-OH D.

e. **Preparation of Quality Control Material**

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 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/mLfor 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 1-mL aliquots into 2.0-mL Nalge cryovials. Cap and label the vials with NHANES barcoded labels that have been specially prepared for the QC pools. Store

the pools at ≤-70 °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.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

INCSTAR prepares Vitamin D calibration standards from HPLC-purified 25-OH-D_3 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.

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 <6% of the CPM of the total counts.

Maximum binding: The CPM of zero ng/mL standard should be >30% 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 addition, the kit standards are run every 6 months as unknowns to verify system calibration.

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) will be performed in order to establish comparability.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. Quick Reference Summary Table

Table 1

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	50µL											
NSB buffer	1 mL	1 mL										
25-OH-D antiserum			1.0 mL									
		Mix well, incubate for 90 min (± 10 min) at 20-25 °C.										
DAG Precipitating Complex		500 µL										
		Mix well; incubate for 20-25 min at 20-25 °C.										
		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.

b. **Extraction Procedure**

(1) Load standards, controls, and samples into a sample rack and place the rack on the Quatro 240.

(2) Label a 12- x 75-mm disposable glass tube for each standard and control and patient sample and place the tubes into the recipient rack.

2) Add 500 µL of acetonitrile to each tube, using the Eppendorf Repeater.

3) Using the Quatro 240, transfer 50 µL 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.

4) After each sample is delivered, place a cork in the top of the tube and vortex the contents for 10 sec.

5) Centrifuge the samples using 1200 x g for 10 min at 20-25 °C.

6) Using the Quatro 240, transfer 25-µL from the supernatant into a second set of appropriately labeled

12- x 75-mm glass reaction tubes in duplicate. (Do not remove the corks until immediately prior to sample transfer.)

CAUTION: Take care not to disturb the pellet.

7) Proceed with the assay procedure.

c. **Assay Procedure**

Follow steps 1-2 to prepare the reaction tubes for analysis:

1) Allow all reagents to equilibrate to ambient room temperature. Do not allow any reagents to warm above

25 °C.

2) Using the Eppendorf Repeater pipet, add reagents as follows:

a) Total Counts Tubes

- 50 µL of ^{125}I 25-OH-D
- 1.0 mL of NSB buffer

b) Nonspecific Binding Tubes

- 25 µL of 0.0 standard (extracted)
- 50 µL of ^{125}I 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 ^{125}I 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 min at 20-25 °C.

4) Using the repeating dispenser, add 500 µL of DAG precipitating complex to all tubes except the total counts tubes.

5) Mix the contents of the tubes well and incubate the tubes for 20-25 min at ambient temperature.

6) Centrifuge using 1800 x g for 20 min at ambient temperature.

7) Decant the supernatants (except the total counts tubes).

8) Using the gamma scintillation counter, count each tube for 1 min to achieve statistical accuracy.

d. **Calculations**

Both the Micromedic Apex and 10/600 Plus counters have full data reduction capabilities. Linear B/B_0 vs $\log_{10}$ concentration with a cubic spline curve fit is used in both counters where:

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

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

This method results in a linearized 6-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. **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 0 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 reassay, 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 reassayed.

The limit of detection, when defined as the apparent concentration at 3 standard deviations from the counts at maximum binding, is 2.78 ng/mL. Any values less than the lowest standard, 5.0 ng/mL, are not calculated by the ISODATA software but 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-D_3 to produce concentrations within specified ranges. Sodium azide is added as an antimicrobial, and the materials are lyophilized for stability.

Like blind QC pools, bench QC pools may also be made from filter-sterilized fasting human serum, as described in Section 6.e. 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 both high-normal and low-normal in-vivo concentrations of QC pools in a matrix identical to that of unknown specimens.

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 labelled in exactly the same fashion as the NHANES III 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.

Long-term estimates of method precision have not yet been established for this method (which was first used in January 1994). Although initial evaluation indicated CVs of 20-30%, it is our experience that once an RIA method is in daily use, CVs may be improved to $\leq$10% fairly quickly.Quality control data is entered into the mainframe ROSCOE program "QCDATA"; the plotting option of "QCPRTA" or "QCGRA" is used to produce long-term plots by using the Division quality control programs. Analysts retain hardcopy updates of these plots; the supervisor maintains

annual files of data and plots.

Limits are established for new pools after 20 runs.

The system is declared "out of control" if any of the following events occur:

For the Means Chart:

A single run mean for one or more pools falls outside the upper or lower 99% limit.
The run means for two or more pools fall either both above or both below the 95% limit.
Two successive run means for a single pool fall either both above or both below the 95% limit.
Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

For the Range Chart:

A single within-run range falls above the upper 99% limit.
The within-run ranges for two or more pools fall above the upper 95% limit.
Two successive within-run ranges for a single pool fall above the upper 95% limit.
Eight successive within-run ranges for a single pool fall above the center line.

Table 2

NHANES III Serum Vitamin D Quality Control Pools

1994 Summary

Pool	Mean (ng/mL)	N	Total Std. Dev.	Total CV (%)	95% Limits	99% Limits
9400	21.6	29	3.23	14.96	15.51-27.62	13.59-29.53
9401	24.9	77	4.53	18.17	16.85-32.95	14.30-35.50
9402	16.1	18	3.10	19.29	10.41-21.72	8.62-23.51
9403	57.0	16	8.17	14.33	41.51-72.59	36.59-77.51
9404	17.2	18	2.97	17.29	11.71-22.65	9.98-24.38
9405	58.9	18	10.72	18.21	39.29-78.47	33.09-84.66
9406	12.0	26	1.63	13.66	9.21-14.70	8.34-15.57
9407	49.3	26	9.11	18.48	32.25-66.28	26.87-71.66
9408	17.7	18	3.10	17.48	11.99-23.51	10.17-25.33
9409	54.7	19	8.60	15.74	39.37-69.95	34.53-74.79
9410	21.6	19	3.48	16.09	15.16-28.07	13.12-30.11
9411	56.8	18	8.04	14.15	41.18-72.45	36.23-77.40
9412	19.5	8	2.94	15.06	13.67-25.37	11.82-27.22
9413	54.3	8	8.09	14.89	38.38-69.97	33.73-74.92

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.

The kit antibody will demonstrate 2.5-100% cross-reactivity with all forms of di-hydroxy-vitamin D_2 and D_3 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 III data will be used to define the normal U.S. levels when statistical analyses of these data are complete.

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 OR STORING SPECIMENS IF TEST SYSTEM FAILS

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

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF

APPLICABLE)

Use the "NHANES Lab Standards and Controls Summary Sheet" for vitamin D to record the quality control data. This reporting sheet has self-explanatory blanks for the standard concentrations and the quality control pool results. Prepare this form in duplicate.

Use the "NHANES III Analytical Worksheet" to record the specimen results. These have been prepared with sample IDs for each preracked run. If a sample is missing from the rack, write "NOSAX" in the blank. If a sample is not satisfactory (i.e., cannot be analyzed), write "UNSAX" in the blank. Prepare these forms in duplicate.

Give both types of forms to the supervisor along with the hard copy of the data printout from the gamma counter computer. After the supervisor checks the data, the carbon copies and the data printouts will be returned for filing in a notebook. The supervisor will keep the original copies of the reporting sheets. The results are transcribed by the data entry clerk into the NHANES III data base that is located in RBASE on the NCEH/EHLS PC network.

Use the "QCDATA6" program in ROSCOE on the CDC mainframe to record quality control data. This program should be updated regularly.

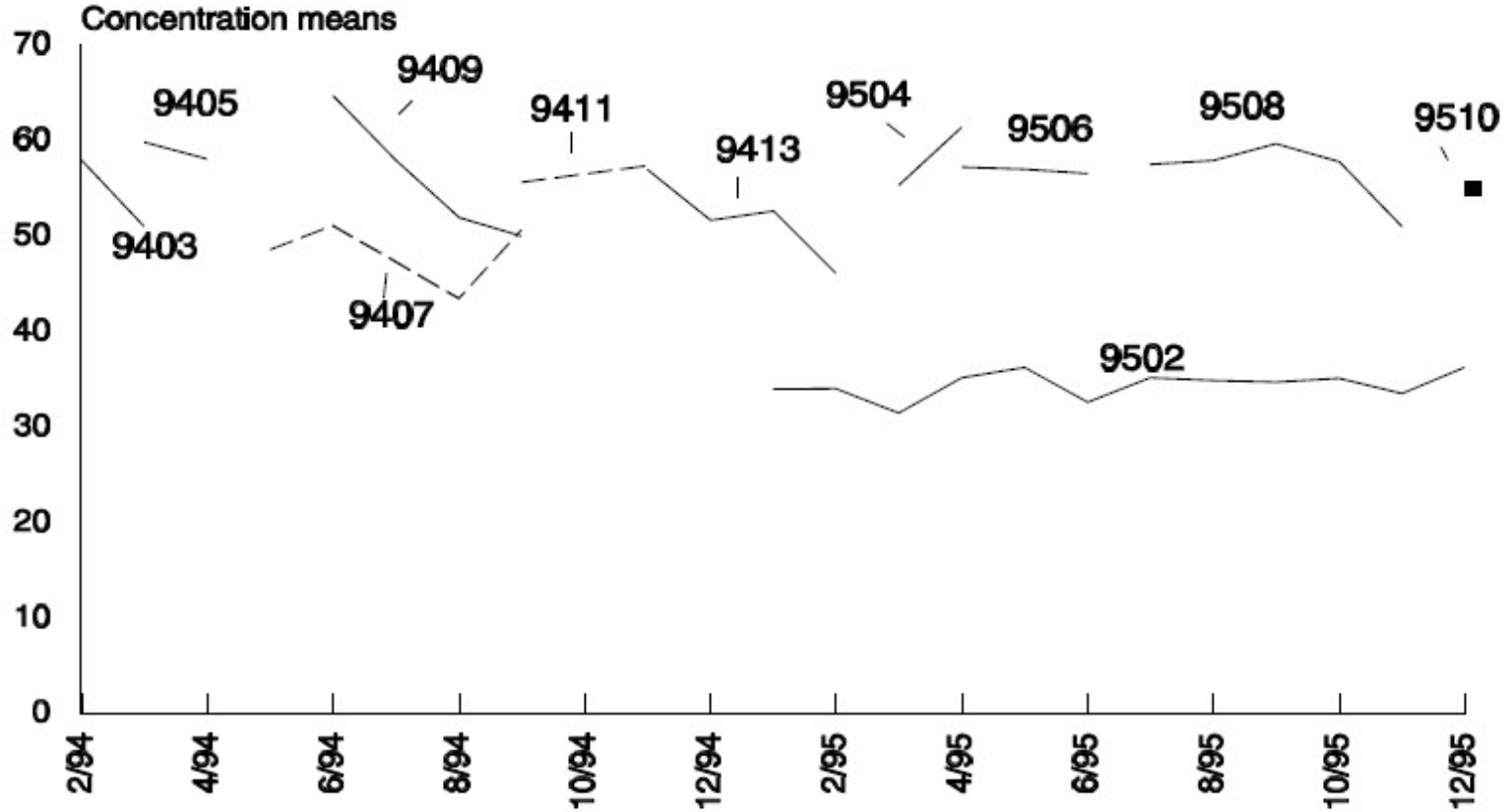
18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

RBASE and the CDC mainframe are used to keep records and track specimens. Records, including related QA/QC data, are maintained for 10 years beyond the duration of the survey. Only numerical identifiers (e.g., case ID numbers) should be used. For the NHANES study, serum remaining after ionized calcium analysis is retained at -70 °C for vitamin D analysis.

19. Summary Statistics and QC Graphs

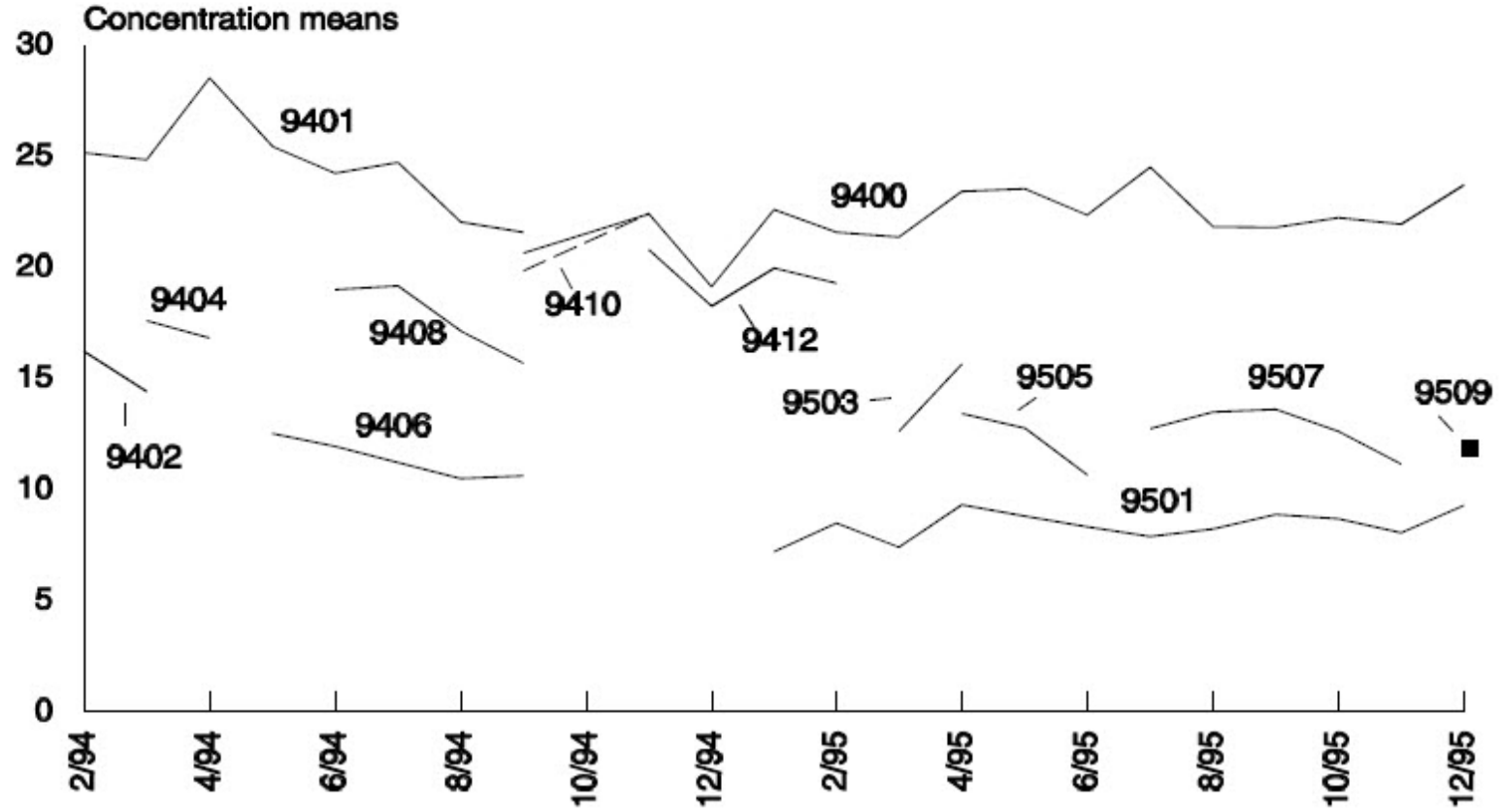
See following pages.

Vitamin D Monthly Means - High Pools



NOTE: Analysis of Vitamin D did not begin until February 1994.

Vitamin D Monthly Means - Low Pools



NOTE: Analysis for Vitamin D did not begin until February 1994.

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