Laboratory Procedure Manual

Analytes: **25-Hydroxyvitamin D_3**
3-epi-25-Hydroxyvitamin D_3
25-Hydroxyvitamin D_2

Matrix: **Serum**

Method: **Ultra High Performance Liquid Chromatography-tandem Mass Spectrometry**

Method No: **4027.03**

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As performed by:

Fat-soluble Nutrients Laboratory
Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences
National Center for Environmental Health

Contact:

James L Pirkle, MD, PhD
Director, Division of Laboratory Sciences

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.

This document details the Lab Protocol for testing the items listed in the following table:

Lab Number	Analyte	SAS Label (and SI units)
VID_G	LBXVIDMS	total 25-hydroxyvitamin D (nmol/L)
	LBXVD2MS	25-hydroxyvitamin D_2 (nmol/L)
	LBXVD3MS	25-hydroxyvitamin D_3 (nmol/L)
	LBXVE3MS	3-epi-25-hydroxyvitamin (nmol/L) D_3

1. Summary of Test Principle, Clinical Relevance

Vitamin D is functionally a hormone rather than a vitamin, and in conjunction with parathyroid hormone and calcitonin, it is one of the most important biological regulators of calcium metabolism. Vitamin D and its main metabolites may be categorized into two families of secosteroids (steroid B-ring open): cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2). Both vitamins D_3 and D_2 are enzymatically hydroxylated in the liver to 25-hydroxy forms and then further metabolized in the kidney to the bioactive 1,25-dihydroxy forms. Conventionally, for vitamin D or any of the relevant metabolites of vitamin D, without a subscript on the "D", the form is not specified and is assumed to include D_2 and D_3. Although 25-hydroxyvitamin D (25OHD) is not the bioactive form, it is the predominant circulating form of vitamin D, and thus, it is considered to be the most reliable index of vitamin D status (1, 2). Vitamin D_3 is a naturally occurring form of vitamin D that is produced in the skin after 7-dehydrocholesterol is exposed to UV-B radiation. Commercially, vitamin D_2 is produced by UV irradiation of plant-derived ergosterol. The two forms differ in the structures of their side chains, but they are metabolized identically. Good sources of vitamin D_3 are fatty fish while mushrooms provide a good source of vitamin D_2. Both forms are used for fortification of a limited selection of foods including milk, juice, margarines, cheese and nutrition bars. Because these two parent compounds provide various contributions to vitamin D status, it is informative when both forms are measured separately (1, 2).

Additionally, $25OHD_3$ exists in at least two isomeric forms that are measurable in serum, 3β-$25OHD_3$ and 3α-$25OHD_3$. The more common 3β isomer is usually referred to as simply $25OHD_3$ while the 3α isomer is usually designated 3-epi-$25OHD_3$. The predominant forms are age-related: $25OHD_3$ in adults and 3-epi-$25OHD_3$ in infants under the age of one year (3). Both C3 isomers of $25OHD_3$ have been observed to coexist in adults. Interestingly, the biological activity of the 1,25-dihydroxy- form of 3α is less than that of its analogous 3β form in several *in vitro* test systems. To summarize, the method described here separates the two C3 isomers allowing for the specific quantitation of the major biological forms (in persons ≥ 1y) of $25OHD_3$ and $25OHD_2$. These are summed to total 25-hydroxyvitamin D (25OHD).

The measurement of 25OHD is becoming increasingly important in the management of patients with various disorders of calcium metabolism associated with rickets, osteomalacia, nutritional and renal osteodystrophy, hypoparathyroidism, and postmenopausal osteoporosis (4-7).

The test principle for the CDC method utilizes ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) for the quantitative detection of 25-hydroxyvitamin D_3 ($25OHD_3$), 3-epi-25-hydroxyvitamin D_3 (epi-$25OHD_3$), and 25-hydroxyvitamin D_2 ($25OHD_2$) in human serum. The analytes are chromatographically separated generally on one of two pentaflurophenyl (PFP) columns (Thermo Scientific Hypersil GOLD PFP 2.1 x 100 mm, 1.9 µm particle size column or Phenomenex Kinetex PFP 2.1 x 100 mm, 1.7 µm). Mobile phase composition for optimized chromatography varies slightly for the two columns, but is between 69% and 72% methanol in water. The composition of the solution added to the serum prior to extraction, the solution used for reconstitution, the needle wash, and the equilibration solution should match that used for the mobile phase.

Serum samples are first treated by the addition of an ethanolic solution containing three internal standards and additional solution of 69-72% methanol to allow enough volume to partition the organic and aqueous phases during extraction; this is followed by the addition of hexane. Analytes are extracted from the aqueous phase into the hexane layer (liquid-liquid extraction), which is then dried under vacuum. The extract is re-dissolved with 69-72% methanol. An aliquot of the extract is injected onto the PFP column for the separation of $25OHD_3$, epi-$25OHD_3$, $25OHD_2$, and the internal standards (IS), 26,26,26,27,27,27-hexadeuterium-25-hydroxyvitamin D_3, 6,19,19-trideuterium-3-epi-25-hydroxyvitamin D_3, and 6,19,19-trideuterium-25-hydroxyvitamin D_2. Detection is performed by using a triple quadrupole tandem mass spectrometer (Thermo TSQ Vantage system) using atmospheric pressure chemical ionization in the positive ion mode. Quantitation is accomplished by comparing the response ratio in the unknowns with the response ratio of a known amount of analyte in a calibrator solution. Response ratios are based on the peak area of the analyte divided by the peak area of the internal standard.

2. Safety Precautions

Consider all serum specimens as potentially positive for infectious agents including HIV, hepatitis B, and hepatitis C. CDC recommends the hepatitis B vaccination series for all analysts working with blood products. Observe universal precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method. Place all disposable plastic, glassware, and paper (such as bench liner, pipette tips, autosampler vials, gloves, etc.) that contact blood products in a biohazard autoclave bag and keep these bags in appropriate covered containers until they are autoclaved. Use disposable bench liners during biological specimen handling and sample preparation, and discard these after use. Also, wipe down all contaminated work surface with 10% bleach solution when work is finished.

Handle organic solvents only in well-ventilated area or, as required, in a chemical fume hood.

Reagents and solvents used in this study include those listed in Section 6. Material safety data sheets (MSDS) 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. During sample preparation and analysis, samples are identified by their Sample ID. The Sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample.

b. The raw data files and respective batch files from the instrument are collected using the instrument software and stored on the local workstation. Raw data are processed into results using the instrument software. The results generated are mostly based on auto-integration by the software, but do allow for manual peak selection and integration when necessary. The final results data file is transferred to the CDC network. The final results data file (including peak areas of analytes and internal

standards, analyte retention times, analyte and internal standard names, dilution factors, data file names, acquisition times, etc) is imported into a LIMS database for review of the data, statistical evaluation of QC/QA data, and approval of the results. See **4027 SOP Computerization and Data System Management** for a step-by-step description of data transfer, review, and approval.

c. For NHANES, data are transmitted electronically on a regular basis (at the end of a survey or approximately weekly for certain 3-week turnaround analytes). Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and transmitted as part of the data file. For those analytes with clinically accepted cutoffs, when possible, NCHS makes arrangements for abnormal report notifications by the NCHS Survey Physician. In some unusual cases, survey lab results are delayed due to major changes in laboratory methods.

d. The instrument raw and results files (including all patient, QC, and calibration data) on instrument computer hard drives are backed up to an external hard drive periodically. This is the responsibility of the analyst under the guidance of the project team leader. Files stored on the network are automatically backed up by CDC ITSO support staff.

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 25OHD 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 100 µL is required for the assay; 500 µL will permit adequate automated pipetting volume and repeat analysis, if necessary.

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 25OHD 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 many 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 interfere (+ or -) with 25OHD results.

h. Specimen handling conditions are outlined in the *Policies and Procedures Manual of the Division of Laboratory Sciences* (copies are available in the Nutritional Laboratory and the

electronic copy of this file is located at \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\CLIA\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. Generally, specimens thawed and refrozen less than five times are not compromised. If there are multiple analytes of interest in the specimen and it needs to be divided for separate assays, the appropriate amount of blood or serum should be transferred into a Nalgene cryovial labeled with a sample ID that reflects a separate aliquot.

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

Not applicable for this procedure

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

a. Reagent Preparation

Any water used to prepare reagents refers to deionized water with resistance of at least 17 megohms. Reagent grade ethanol and HPLC-grade hexane and methanol are used throughout.

1) HPLC Mobile Phase – 69-72% Methanol

Obtain 2 clean dry graduated cylinders. To one cylinder add 280-310 mL water and to the other cylinder add 690-720 mL methanol, depending on desired solvent composition. Pour both solutions into a 1000-mL bottle. Swirl the solution to mix then ideally, place the mobile phase bottle in a sonicator for at least 30 minutes to remove excess gas. This solution is stable at room temperature, so prepare as needed.

2) HPLC Mobile Phase - 100% Methanol

Methanol is added to a 1000-mL bottle. This solution is stable at room temperature, so prepare as needed.

3) HPLC Needle Wash – 69-72% Methanol

Match needle wash composition to that of mobile phase. Methanol (690-720 mL) is added to a 1000-mL bottle. Water (280-310 mL) is added to the same bottle. Ideally, place the needle wash bottle in a sonicator for at least 30 minutes to remove excess gas. This solution is stable at room temperature, so prepare as needed.

4) 0.01M Phosphate-buffered saline (PBS); pH 7.4

Sigma catalog number P-3813; 1 packet is dissolved in 1 liter of water. This solution is stable at room temperature and is used for standard solutions.

5) 4% Albumin in 0.01M PBS

Weight out 2 grams of albumin and dissolve in 50 mL of 0.01M phosphate buffer saline. Vortex and stir it on a magnetic stirrer until it totally dissolved. Store this solution in a refrigerator at 4˚C. Prepare fresh every two weeks.

b. Standards Preparation

The stock solutions and calibration solutions are stored in 1.8-mL polypropylene cryovials at -70˚C and are stable for over a year.

1) ~25 µmol/L Stock Solutions

Prepare **25OHD$_3$** Stock I by dissolving ~1 mg 25OHD$_3$ in 100% filtered ethanol (filtered through a 0.45-µm filter into a 100-mL volumetric flask). Bring to volume with ethanol and mix.

Prepare **epi-25OHD$_3$** Stock I by dissolving ~1 mg epi-25OHD$_3$ in 100% filtered ethanol (filtered through a 0.45-µm filter into a 100-mL volumetric flask). Bring to volume with ethanol and mix.

Prepare **25OHD$_2$** Stock I by dissolving ~1 mg 25OHD$_2$ in 100% filtered ethanol (filtered through a 0.45-µm filter into a 100-mL volumetric flask). Bring to volume with ethanol and mix.

Using a calibrated UV/vis spectrophotometer, measure the absorbance (AU) of each stock solution at 264nm using ethanol as a reference blank. Calculate the actual concentrations of 25OHD$_3$ and 25OHD$_2$ in each stock solution by applying the following equation: $\mathbf{AU = c^*\varepsilon_{264}^*\ell}$

AU is defined as absorbance units
c is the concentration
ε_{264} is the extinction coefficient of all three analytes at 264nm = 18,200 L/mol*cm
ℓ is the path length, which is 1 cm for standard cuvettes

For nmol/L, the calculation for concentration is identical for 25OHD$_2$, 25OHD$_3$, and 3-epi-25OHD$_3$ where $c_{nmol/L}$=AU/18,200 L/mol*cm * E^9 (or $c_{nmol/L}$ = AU*54,945).

For ng/mL, on the other hand, the extinction coefficients must be adjusted by the atomic mass (AMU) of each metabolite, 25OHD$_{2\ AMU}$=412.7 g/mol and, both 25OHD$_3$, and 3-epi-25OHD$_3$ AMU=401.6 g/mol.

The conversion for ε_{264} is (18,200L/mol*cm ÷ AMU g/mol) ÷ E^5. Hence:

ε_{264} (25OHD$_2$) = 4.40998E^{-5} mL/ng*cm
ε_{264} (25OHD$_3$ and 3-epi-25OHD$_3$) = 4.53187E^{-5} mL/ng*cm

2) Calibration Solutions

Calibration solutions are prepared by mixing the appropriate volume of each stock solution with 70% methanol/water or 4% albumin in PBS in Class A volumetric flasks. The highest calibrators are prepared in 70% methanol/water and all

subsequent calibrators are made via the indicated dilutions (Table 1) with either 70% methanol/water or 4% albumin in PBS. Each lot will vary in concentration. Each calibrator is thoroughly vortexed and stored at -70ºC until use.

Table 1: Summary of typical calibrator preparation

Calibrator ID	Volumes blended for each calibrator					Final concentration*		
	volume $25OHD_2$ stock	volume $25OHD_3$ stock	volume epi-$25OHD_3$ stock	calibrator volume	volume 4% albumin PBS solution or 70% methanol	$25OHD_2$ (nmol/L)	$25OHD_3$ (nmol/L)	epi-$25OHD_3$ (nmol/L)
Stock concentration	-	-	-	-	-	38132.8	42442.8	38922.8
Cal 6	214.4 µL	385.2 µL	168.0 µL	-	108.2 mL	75.0	150	60.0
Cal 5	194.7 µL	238.6 µL	156.1 µL	-	134.4 mL	55.0	75.0	45.0
Cal 4	-	-	-	52.8mL of Cal 5	35.2 mL	33.0	45.0	27.0
Cal 3	-	-	-	22.6 mL of Cal 6	90.4 mL	15.0	30.0	12.0
Cal 2	-	-	-	28.0 mL of Cal 3	56.0 mL	5.00	10.0	4.00
Cal 1	-	-	-	5.4 mL of Cal 4	75.6 mL	2.20	3.00	1.80
Blank	-	-	-	-	20 mL**	0	0	0

*Final concentrations of calibrators shown are the theoretical target values. Calibrator values are re-assigned after preparation based upon harmonization with SRM materials.

**Blank is always made with 4% albumin-PBS

3) Internal Standard Solutions (Stock and Working)

The internal standards arrive from the vendors in powder form at ambient temperature.

d6-25OHD3: each vial contains 1 mg of 26, 26, 26, 27, 27,27-hexadeuterium-25-hydroxyvitamin D_3. Add 20 mL of ethanol into the vial and vortex well; this is a 0.05 mg/mL stock solution. The material is stored at -70°C.

d3-$25OHD_2$: each vial contains 1 mg of 6,19,19-trideuterium-25-hydroxyvitamin D_2. Add 40 mL of ethanol into the vial and vortex well; this is a 0.025 mg/mL stock solution. The material is stored at -70°C.

d3-epi-$25OHD_3$: the vial contains 1 mg of 6, 19, 19-trideuterium-3-epi-25-hydroxyvitamin D_3. Add 20 mL of ethanol into the vial and vortex well; this is a 0.050 mg/mL stock solution. The material is stored at -70°C.

A working internal standard solution is made by blending the three stock solutions together using a 66% ethanol in water solution as diluent to obtain a final concentration of 75 nmol/L *d6*-$25OHD_3$, 25 nmol/L *d3*-$25OHD_2$, and 25 nmol/L *d3*-3-epi-$25OHD_3$.

c. Preparation of Quality Control Materials

Low, medium, and high quality control bench pools are prepared from pooled human serum obtained from blood bank donors with high or low serum 25OHD levels. Target

levels are sought for the individual analytes in each of the three levels, about 14-63 nmol/L for $25OHD_2$ and 30-86 nmol/L for $25OHD_3$.

To prepare pools, first prescreen units for $25OHD_3$ and $25OHD_2$ concentrations. Calculate blends of serum to achieve at least 500 vials each of low, medium and high pools based on screening values. Gravity-filter the serum through several layers of sterile gauze. For each pool, blend the serum in an acid-cleaned 1-liter glass bottle and mix well on a magnetic stirrer. Using sterile technique under a laminar-flow hood, dispense the continuously-mixed serum in 500-µL aliquots into 2.0-mL Nalgene cryovials. Select twenty vials of each level at random for characterization of quality control limits and for testing of homogeneity. Store the pools at ≤ -70˚C. Note, sometimes it is necessary to spike serum with analytes to achieve the desired concentrations.

Two levels of blind QC pools may be prepared in the same way that bench pools are prepared. Store the pools at ≤ -70˚C. These pools are inserted randomly into the NHANES runs at 1 blind QC vial in every 20 participant's specimens. Select twenty vials of each level at random for characterization of the blind QC limits and for testing of homogeneity. Note, small studies often do not use blind QC.

d. Other Materials

1) Kinetex pentaflurophenyl (PFP) 2.1 x 100 mm; 1.7 µm particle size column (Phenomenex, Torrance, CA)
2) Hypersil GOLD pentaflurophenyl (PFP) 2.1 x 100 mm; 1.9 µm particle size column (Thermo Scientific, West Palm Beach, FL)
3) KrudKatcher Ultra Inline HPLC filter, 0.5µm depth x 0.004in ID (Phenomenex, Torrance CA)
4) 2.1mm IDx2µm inline filter (Thermo Scientific, Belefonte, PA)
5) 13 x 100 mm Disposable glass culture tubes (Corning Glassworks, Corning, NY)
6) 5¾" Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)
7) Solvent filters, 0.45µm pore size (Millipore Corp, Medford, MA)
8) N-Dex nitrile examination gloves (Best Manufacturing Corp, Menlo, GA)
9) 0.45µm Syringe tip PVDF hydrophilic filter (4 mm diameter) (obtained from various sources)
10) Plastic tuberculin syringes (obtained from various sources)
11) 1.8-mL Polypropylene cryovials (Nalgene Company, Rochester, NY)
12) Various glass beakers, volumetric flasks, graduated cylinders and bottles (class A glassware)
13) Methanol, HPLC grade (Tedia, Fairfield, OH)
14) Ethanol, HPLC grade (obtained from various sources)
15) Albumin from bovine serum (Sigma, St. Louis, MO)
16) 25-Hydroxyvitamin D_3 (USP, Rockville, MD; Sigma, St. Louis, MO)

17) 25-Hydroxyvitamin D_2 (Isosciences, King of Prussia, PA; Sigma, St. Louis, MO)
18) 3-Epi-25-Hydroxyvitamin D_3 (Isosciences, King of Prussia , PA)
19) 26,27-Hexadeuterium-25-hydroxyvitamin D_3 (Medical Isotopes Inc, Pelham, NH)
20) 6,19-Trideuterium-25-hydroxyvitamin D_2 (Isosciences, King of Prussia, Pa.)
21) 6,19-Trideuterium-3-Epi-25-hydroxyvitamin D_3 (Medical Isotopes Inc, Pelham, NH)
22) Rainin pipette tips, 200- and 1000-µL (Rainin Instrument, LLC, Woburn, MA)
23) Gilson Microman positive displacement pipette tips, 100 µL and 250 µL (Gilson, Villiers-le, France)
24) Parafilm, 4-inch wide roll (any vendor)
25) 96-Cell round bottom well plates, 1.2-mL (Fisher Scientific, Pittsburg, PA)
26) Preslit silicone plate seals 8.6 mm (Fisher Scientific, Pittsburg, PA)
27) Hamilton Robotic liquid handler 300-µL and 1000-µL tips (Hamilton, Reno, NV)

e. Instrumentation

1) Thermo Vantage mass spectrometer, with Xcalibur software (ThermoElectron Corp, West Palm Beach, FL) or comparable
2) Thermo Accela UHPLC system (ThermoElectron Corp) or comparable
3) Nitrogen generator, model NM30L-MS (Peak Scientific Instruments, Chicago, IL)
4) Rheodyne 2-position, 6-port fluid switching valve (ThermoElectron Corp)
5) Cary 3E spectrophotometer (Varian Instruments, Palo Alto, CA) or comparable
6) Gilson Microman positive displacement pipettes, 100 µL and 250 µL (Gilson, Villiers-le, France)
7) Rainin pipettes (2-20µL, 100-250µL, and 100-1000µL) (Rainin Instrument, LLC, Woburn, MA)
8) Single tube and multitube vortexers (VWR, Suwanee, GA)
9) Digiflex dispenser (Titertek, Huntsville, AL) or comparable
10) Magnetic stirrer (Fisher Scientific Co., Fairlawn, NJ)
11) Mettler Toledo analytical balance, model AG104 (Mettler Instrument Corp, Hightstown, NJ)
12) Eppendorf 5810R Centrifuge (Eppendorf, Westbury, NY)
13) Speedvac SC200 and SC210A Systems (Savant Instrument Co, Farmingdale, NY) or equivalent
14) Precision model VP-190 direct drive vacuum pump (Precision Scientific Inc, Chicago, IL) or equivalent
15) Refrigerated vapor trap, model RVT-4104 (Savant Instrument Co) or equivalent
16) Hamilton Microlab STARlet (Hamilton, Reno, NV) or comparable

7. Calibration and Calibration Verification Procedures

Instrument
The calibration of the instrument is scheduled on an annual basis, as part of the preventive maintenance, and is performed by the service engineer from ThermoElectron Corp. If necessary, the analyst recalibrates using the calibration standards described below and by following the instructions contained in the Operator's Manual. See **4027 SOP_MS Tuning & Optimization_Thermo LCMSMS** for detailed instructions.

Compound-dependent optimization of instrument (TSQ Vantage) is generally done initially when setting up the method on a new instrument. Analysts may periodically conduct optimizations when major service is conducted. See **4027 SOP_MS Tuning & Optimization_Thermo LCMSMS** for detailed instructions.

Assay
At the beginning of each run, six mixed calibrators (containing 25OHD$_3$, epi-25OHD$_3$, and 25OHD$_2$) with concentrations ranging from about 2 to 150 nmol/L are prepared as described in section 6.b. of this document. Two calibration preparation techniques have been validated for use by this method using either direct injection or extraction of the calibrators. Any one of the following techniques are appropriate:

Direct injection - solvent based calibration:
This method uses 70% methanol/water as the matrix for the calibrators. The need for a carrier protein as part of the calibration matrix was found to be unnecessary in 2013. Solvent-based calibration was compared to the original matrix-based calibration (4% albumin in PBS) and found to be comparable and produced satisfactory results based on NIST reference materials. Prior to that, albumin-based calibration was tested by comparing the average slopes of three 10-point calibration curves prepared using serum (un-stripped) as the matrix with three 10-point calibration curves using 4% albumin in PBS. For all three analytes, <5% difference in the average calibration curve slopes was observed between calibrators containing serum and albumin-PBS. The differences observed were of a similar magnitude to slope variability observed within and between individual calibration curves of a particular matrix. For this method, a 100-µL aliquot calibrator is mixed with a 75-µL of internal standard solution with no further processing.

Extracted calibration - 4% albumin-PBS matrix:
While the method does not require a carrier protein, day-to-day precision may be more than expected when the calibrators are prepared using solvent-based materials. Taking matrix-based calibration materials through the extraction process improves precision. In this process, a 100-µL aliquot of calibrator is mixed with a 75-µL aliquot of internal standard solution and 100 µL of mobile phase. The resulting solution is carried through the full extraction process.

Irrespective of the exact technique, the calibration process is based on the peak area ratios of each of the individual vitamin D metabolites compared with its internal standard, also known as response ratio, as follows: 25OHD$_3$/ *d6*-25OHD$_3$, epi-25OHD$_3$/ *d3*-epi-25OHD$_3$, and 25OHD$_2$/ *d3*-25OHD$_2$. Six-point linear curves, not forced through zero, are generated. The concentrations (x-axis) are calculated from the regression equation based on the response ratios of each (y-axis). Calibration solutions are carried through

the sample preparation procedure. Curves are weighted as follows: $25OHD_3$ (1/x); $25OHD_2$ ($1/x^2$); epi-$25OHD_3$ ($1/x^2$).

If the test system calibration procedure includes: 1) three or more levels of calibration materials; 2) a low point near the LOD, mid, and high values; and 3) and is performed at least once every six months, then the requirement for calibration verification is also met.

The CDC laboratory participates in four proficiency testing programs for 25OHD. One is sponsored by DEQAS (Vitamin D External Quality Assessment Scheme); another is sponsored by National Institutes of Standards and Technology (NIST, Gaithersburg, MD). The others are the CAP Bone and Growth Survey and the CAP Accuracy-based Vitamin D Survey. Every three months five specimens are sent by DEQAS, twice a year three specimens are sent by NIST, and twice a year 2-4 specimens are sent by CAP to assess laboratory performance. In addition, NIST makes available 4 levels of Standard Reference Materials (SRM) for serum $25OHD_3$, 3-epi-$25OHD_3$, and $25OHD_2$, containing certified and uncertified values; these materials are tested at least four times a year. In addition, NIST also provides certified ethanolic SRM 2972 for $25OHD_3$ and $25OHD_2$ and these are used to verify our stock standard solution concentrations at least twice a year; see **Calibration Verification SOP for VID UPLCMSMS_4027_v2** for additional details.

To provide adequate throughput for this method, as well as backup instrumentation during times of repair and maintenance, we utilize multiple LC-MS/MS systems of the Thermo Vantage type. Equivalent performance (system verifications) was demonstrated in accordance with CDC *DLS Policies and Procedures Manual* when multiple analysis systems were used in parallel, even if they are of the exact same type. The comparisons involved analyzing several samples on each of the instruments and assessing the resulting Pearson correlation coefficients. Details about these procedures can be found in **Calibration Verification SOP for VID UPLCMSMS_4027_v2.**

Thorough method validation was performed on this method and corresponding figures of merit may be seen for method performance in **Appendix A** and ruggedness in **Appendix B**.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

a. Sample Preparation (automated liquid handler)

1) Remove all necessary QC, calibrators, patient samples, and the mixed internal standard solution from the -70°C freezer. Allow them to reach ambient temperature then gently vortex prior to pipetting. Always visually check for any unusual sample volume, specimen color or debris/precipitate.

2) Use 4% albumin-PBS solution as the blank.

3) An automated liquid handler (Hamilton Microlab STARlet) should be preprogrammed for the procedure. For programming instructions see Hamilton Microlab STARlet Operators Manual and see **4027 SOP Automated Sample Pipetting for VitD LCMSMS By Hamilton** for detailed method specific instructions.

4) *Extraction Phase*:

   a) Step 1: Transfer 100 µL of QC, matrix-based calibrator, or patient serum sample to thin walled 13 x100 mm borosilicate tubes.

   b) Step 2: Transfer 75 µL of the IS solution to each tube and allow robotic mixing.

   c) Step 3: Transfer 100 µL of 69% or 72% (column-dependent) methanol to each tube and allow robotic mixing.

   d) Step 4: Transfer 1.5 mL hexane to each tube. No robotic mixing is done at this step since the tubes will be manually vortexed.

   e) Step 5: Shake the 13 x 100 mm tubes containing the above mixture using a multi-tube vortexer at 1,600 setting for 3 minutes. Allow a 1-minute rest period before repeating the process. Repeat again for a total of 3 shake steps.

   f) Step 6: Centrifuge the shaken tubes for 5 minutes at 3,000 rpm to break up any emulsions that may have formed during shaking.

   Reconstitution Phase:

   g) Step 7: Robotically transfer 1 mL of the hexane layer from each tube to the corresponding position in the 96-well plate.

   h) Step 8: Dry down the well plate via Turbovap, Speedvac, or a nitrogen plate dryer to evaporate the hexane completely. If using a Speedvac, set the instrument to the highest vacuum with no heat. If using a plate dryer, dry under nitrogen at 35 L/min.

   i) Step 9: Add 300 µL of 69-72% methanol to each dried cell.

   j) Step 10: Gently shake for 10 minutes on a plate shaker.

5) Cover the plate with a preslit silicone plate cover and load into the Accela autosampler chamber set at 7°C.

6) If preparing solvent-based calibrators for direct injection: calibrators are prepared by aliquotting a 100µL of the standard into a well on the 96-well plate, then aliquotting 75µL of the IS to that well and vortexing.

b. LC-MS/MS Analysis

1) The analytes are eluted from the PFP analytical column held at 28^{o}C under isocratic conditions of 69% methanol: 31%water (Thermo column) or 72% methanol: 28%water (Phenomenex column) at flow rate of 400 µL/min.

2) $25OHD_3$, 3-epi-$25OHD_3$, $25OHD_2$, 26,27-hexadeuterium-25-hydroxyvitamin D_3 ($25OHD_3$-IS), 6,19-trideuterium-25-hydroxyvitamin D_2 ($25OHD_2$-IS), and 6,19-trideuterium-3-Epi 25-hydroxyvitamin D_3 (epi-$25OHD_3$-IS) are detected by using MS/MS on a TSQ Vantage system and atmospheric pressure chemical ionization (APCI) in the positive ion mode.

3) *Quantitation Ions*: The following transitions are recorded (the dehydrated molecular ion is the parent ion, and the 2nd loss of water is the daughter ion):

$25OHD_3$, *m/z* 383.3→365.3; **epi-$25OHD_3$**, *m/z* 383.3→365.3; **$25OHD_2$**, *m/z* 395.3→377.3; Internal Standards: ***d6*-$25OHD_3$** *m/z* 389.3→371.3, ***d3*-epi-$25OHD_3$** *m/z* 386.3→368.3, ***d3*-25OHD2** *m/z* 398.3→380.3. The elution order of the analytes is $25OHD_3$, epi-$25OHD_3$, and then $25OHD_2$ with the internal standard eluting at the same time as its corresponding unlabeled analyte. The retention times are variable from run to run but the elution times occur in <11 minutes.

Qualitative (Confirmation) Ions: Alternative product ions are measured to confirm peak identity. The ratio of the area of the quantitative ion ÷ the area of the qualitative ion is monitored. The following qualitative transitions are recorded: **$25OHD_3$**, *m/z* 383.3→105.1; **epi-$25OHD_3$**, *m/z* 383.3→105.1; **$25OHD_2$**, *m/z* 395.3→209.1. No confirmation ions are monitored for the internal standards. In addition, each assay is calibrated for the qualitative ions. The ratio of the concentration results using the different ions is checked for agreement. More detailed information about the rules used for confirmation of peak identity is provided in **Appendix C**.

4) The MS instrument settings are as follows: Currents: corona current =7.0 µA; Voltages: Collision energy = 16V, Declustering voltage = -8V, S-lens voltage = 103 V for quantitative ions, 85 V for qualitative ions; Temperatures: Capillary temperature = 170°C, Desolvation/Vaporizor temperature = 400°C; Pressures: Collision pressure = 1.2 mTorr, Sheath gas pressure = 20 psi, Ion sweep gas pressure = 0 psi, Auxillary pressure = 5 psi.

5) A portion (50 µL) of the sample is injected. The first 5 min of each injection is diverted to waste, data are collected from 5-10 min, and the effluent is again directed to waste for the remainder of the run. There is a 2 min wash with 100% methanol directly following data collection. Each injection takes 14-17 min to finish.

c. Instrument Preparation

1) UHPLC Preparation

   a. Refer to **4027 SOP VitD LCMSMS Instrument preparation** for detailed instructions

   b. Mobile Phase Solvents: Line A 69-72% methanol, and Line B 100% methanol

   c. Needle Wash solution: 69%-72% methanol

d. Replace PFP analytical column as needed. Generally, a column will need to be replaced when the column back pressure is high enough to cause the pump to shut off during a run or when peak resolution declines

2) Mass Spectrometer Preparation

a. Refer to **4027 SOP VitD LCMSMS Instrument preparation** for detailed instructions on preparing the instrument for analysis, shutdown and restart procedures, and cleaning procedures.

d. Run Samples on the LC-MS/MS

1) See **4027 SOP VitD LCMSMS Instrument preparation** for detailed information on building a run sequence.

2) Individual run sequences are produced for each 96-well plate. Once the data are collected, they are part of the run sequence.

e. Quantitation

1) Refer to **4027 SOP VitD LCMSMS Instrument preparation** for detailed instructions for using Thermo XCalibur software to perform integrations and quantitation.

2) Export the run to Excel then import to the laboratory information management system database (NBB_DB or NBB_LIMS) for review.

f. Data Review

Refer to **4027 SOP VitD Thermo LCMSMS Computerization & Data system management** for detailed information on data handling using the LIMS.

Check calibration curves for each analyte. Correlation coefficients should be $R > 0.99$. Review each chromatogram and do manual peak selection and integration when necessary.

Check bench QC results for each analyte against QC limits. If any run mean is outside 3SD, or 2 or more of 3 run means (L, M, and H) are outside the same 2SD limit, reject the run for that analyte. This failure means that the run must be repeated. See section 10.b. for bench QC rules.

For each study, a QC results table and QC plots are generated using Excel and a SAS program, respectively. The QC results and plots are reviewed by supervisor.

Print hard copies of the results of integration and quantitation for a sample or a set of samples, only if needed for documentation of unusual occurrences. Generally, hard copies are not needed. Electronic files of the runs are saved for documentation. The LIMS is backed-up regularly.

All results are checked and reviewed by supervisor before sending the final results to the study principal investigator.

g. System Maintenance

1) Thermo TSQ Vantage – Preventative maintenance is performed annually or semi-annually by an authorized service engineer. Analyst performs maintenance as described in **4027 SOP VitD LCMSMS Instrument preparation** as needed due to dropping sensitivity or signal loss. Analyst may also setup a schedule for certain operations such as cleaning various components, ballast pumps, etc.
2) Accela Plus UHPLC system – Preventative maintenance is performed annually or semi-annually by an authorized service engineer. Analyst performs maintenance as described in **4027 SOP VitD LCMSMS Instrument preparation** as needed due to sample delivery problems.
3) Cary 3E Spectrophotometer – Preventative maintenance and calibration of the instrument are performed annually by an authorized service engineer. Calibration verification is performed every six months using internal diagnostics and a set of certified filters. Proficiency testing is provided through the CAP Instrument Survey.

h. CDC Modifications

This method was published in 2011 (10). This document represents the third version of the official method for the CDC lab.

9. Reportable Range of Results

The method described here is designed to detect serum $25OHD_2$ and $25OHD_3$ isomers at values from approximately 2-150 nmol/L. When $25OHD_3$ values are <12.5 nmol/L, the results are verified by re-analysis. There is no threshold level for repeats for $25OHD_2$ or epi-$25OHD_3$. Values greater than the highest calibrator are diluted with PBS-4% albumin and confirmed through repeat testing. The difference between repeat values should generally be within 15% for $25OHD_3$ and if the concentrations are high enough, about 20% for epi-$25OHD_3$ and 25-OHD_2. Otherwise, another repeat needs to be done.

The reportable ranges of serum concentrations are as follows:

$25OHD_3$	2.23 - 150 nmol/L
3-epi-$25OHD_3$	1.64 - 60.0 nmol/L
$25OHD_2$	2.05 - 75.0 nmol/L

10. Quality Control (QC) Procedures

a. Blind Quality Controls

Blind QC specimens are prepared using two serum pools that emulate a low and a high level of serum 25OHD in patient samples. High levels may be achieved by spiking. Samples from these pools are prepared in the same manner as patient samples. For most studies, blind controls are inserted prior to the arrival of the samples to the Nutritional Biomarkers Branch and the labels are identical to these used in the study. Starting in 2012, an open label blind QC program was instituted. Open label blind QC specimens can be used where the analyst knows that the sample is a blind QC, but the analyst does not know to which pool the sample belongs. Open label blind QCs are only used if at least 6 different pools are simultaneously available and the analyte concentrations are similar to those found in patient samples.

b. Bench Quality Controls

Bench QC specimens are prepared using three serum pools that represent low, medium, and high levels of serum 25OHD. Samples from these pools are prepared in the same manner as patient samples and analyzed in duplicates (placed at the beginning and end of each run).

The results from the pools are checked after each run. The system is declared "in control" if all individual QC results are within 2s limits, and the run is accepted. If not then apply rules below and reject if any condition is met - the run is then declared "out of control":

I. One QC result per pool (due to accidental loss of duplicate)

If one of the three QC run **individual** results is outside a 2 S_i limit, reject run if:

(a) 1 3S Rule—Run result is outside a 3 S_i limit or
(b) 2 2S Rule—Two or more of the three run results are outside the same 2 S_i limit
(c) 10 Xbar Rule—Current and previous nine run results are on the same side of the characterization mean or
(d) R 4S Rule—Two consecutive standardized run results differ by more than 4 S_i

II. Two QC results per pool (all three pools have duplicate results)

If one of the three QC run **means** is outside a 2 S_m limit, reject run if:

(a) 1 3S Rule—Run mean is outside a 3 S_m limit or
(b) 2 2S Rule—Two or more of the three run means are outside the same 2 S_m limit or
(c) 10 Xbar Rule—Current and previous nine run means are on the same side of the characterization mean

If one of the six QC **individual** results is outside a 2 S_i limit, reject run if:

(a) Outlier—One individual result is beyond the characterization mean ± 4 S_i or
(b) R 4S Rule—Two or more of the within-run ranges in the same run exceed 4 S_w (i.e. 95 percent range limit)

Abbreviations:
S_i = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements)
S_m = Standard deviation of the run means (the limits are shown on the chart)
S_w = Within-run standard deviation (the limits are not shown on the chart)

A QC program written in SAS (11) 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 using bench QC.

The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated periodically. When necessary, limits are updated to include more runs.

While a study is in progress, QC results are stored in the LIMS database. For runs that are not imported into the database (e.g., analytical method research runs), QC results are stored electronically in the analyte-specific folder on the CDC network. At the conclusion of studies, complete QC records are prepared for review by a DLS statistician.

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

a. Check to make sure that the hardware is functioning properly. Make sure the mass spectrometer calibrations are proper. Run 1, 3, 6 polytyrosine solution to check the instrument calibration. Determine if the mass spectrometer sensitivity is adequate.
b. Run standards to see if the molecular ion is detected.
c. Check for proper gas flow from the nitrogen generator.
d. Check the autosampler for evidence of correct sample injections. Check statistical repeatability of multiple injections.
e. Check column for adequate separation.
f. Look for sample preparation errors, e.g., added internal standard, specimen, etc.
g. Check the calibrations of the pipettes and robotic liquid handler.
h. If the steps outlined above do not result in correction of the "out of control" values for QC materials, consult the supervisor for other appropriate corrective actions.

i. Do not report analytical results for runs not in statistical control.

12. Limitations of Method; Interfering Substances and Conditions

The most common cause of imprecision is pipetting errors. Other sources of procedural imprecision may be the extraction step and contamination originating from the robot such as contaminated solvent reservoirs or dripping channels.

This method has undergone a series of in-house ruggedness testing experiments designed to assess by how much the results change when certain experimental parameters are varied. Two parameters judged to most likely affect the accuracy of the method have been identified and tested. Testing generally consisted of performing replicate measurements on a specimen with the selected parameter set at a value substantially lower or higher than that specified in the method while holding all other variables constant. Ruggedness findings for this method are presented in **Appendix B.** Refer to the latest *DLS Policies and Procedures Manual* for further information on ruggedness testing. **Appendix C** details rules used to assess peak identity.

13. Reference Ranges (Normal Values)

Season, race (skin darkness), latitude, sun protection behaviors, and diet and supplement intake are all known to affect the levels of 25OHD. The reported difference in 25OHD values attributable to seasonal variation in ultraviolet radiation illustrates the importance of personal exposure to sunlight (8, 9). The highest levels of 25OHD are found during the summer to fall months, and the lowest levels during late winter and early spring.

Table 2 shows the 2011 Institute of Medicine (IOM) determination of the health status associated with various serum concentrations of total 25-hydroxyvitamin D (13). Levels less than 30 nmol/L are associated with increased risk of deficiency.

Table 2. Serum 25-hydroxyvitamin D 25 concentrations and health

Serum 25OHD (nmol/L)	Health status
< 30	Associated with vitamin D deficiency, leading to rickets in infants and children and osteomalacia in adults
30 - 50	Generally considered inadequate for bone and overall health in healthy individuals
≥ 50	Generally considered adequate for bone and overall health in healthy individuals
> 125	Emerging evidence links potential adverse effects to such high levels, particularly >150 nmol/L (>60 ng/mL)

14. Critical Call Results ("Panic Values")

At this time, this method is used to collect vitamin D metabolite data for national prevalence purposes only. NHANES 2007-2012 vitamin D metabolite testing was substantially delayed due to a major change in the analytical method. Unlike the previous method, the current method is traceable to certified reference materials.

15. Specimen Storage and Handling during Testing

Specimens should be brought to room temperature before aliquoting for testing, and then be promptly refrozen for storage.

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

There are no suitable alternative methods for the analysis of epi-25OHD_3 in the Nutritional Biomarkers labs. There are alternative methods for the analysis of 25OHD_3 and 25OHD_2 but these do not separate the 3-epimer of 35OHD_3. Thus, the current LC-MS/MS method is preferred. The analyst should store all processed specimens at 4^{o}C for up to two weeks or at ≤ -20°C for longer storage until the system is once again 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 using 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 xlsx or csv file through electronic mail or via FTP. For NHANES, all data are reported electronically to the Westat ISIS computers. For some smaller studies, hard copies of a data report are sent, as well as the results in electronic format.

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

A LIMS database is used to keep records and track specimens for this analytical method. Records, including related QA/QC data, should be maintained for 10 years after completion of the study. Only numerical identifiers should be used (e.g., Sample ID). All personal identifiers should be available only to the medical supervisor or project coordinator. At the request of the principal investigator, residual serum from these analyses for non-NHANES studies may be discarded or may be transferred to the CDC CASPIR facility for use by other investigators. Very little residual material will be available after NHANES analyses are completed, and these vials may be routinely autoclaved.

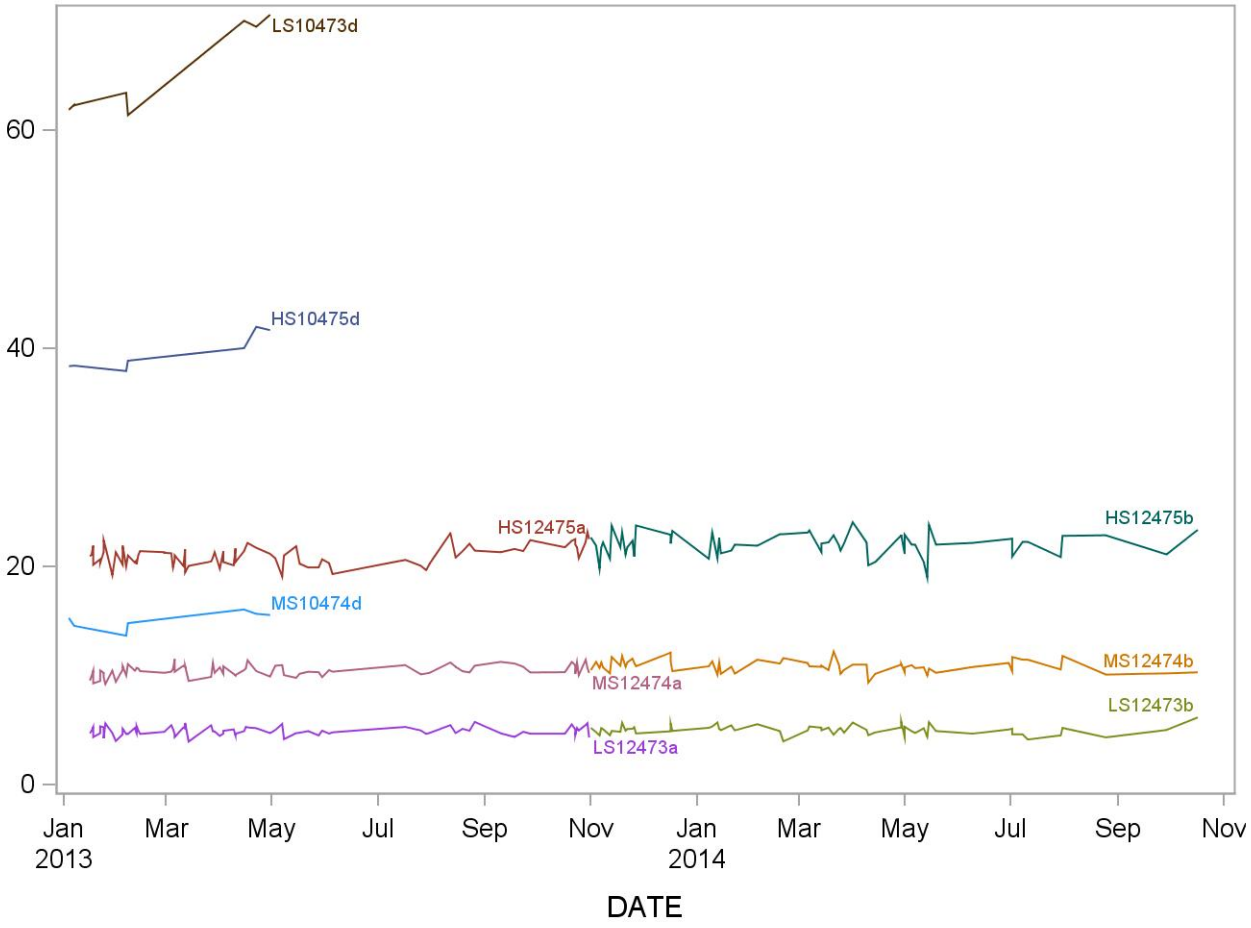
The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the Sample ID number is entered into a database and the specimens stored in a freezer at -70°C. The Sample ID is read off of the vial by a barcode reader used to prepare the electronic specimen table for the analytical system. The analyst is responsible for updating the database with comment codes describing the ID numbers of specimens prepared incorrectly, or those with labeling problems, or those with abnormal results, together with information about these discrepancies.

19. Summary Statistics and QC Graphs

See following pages

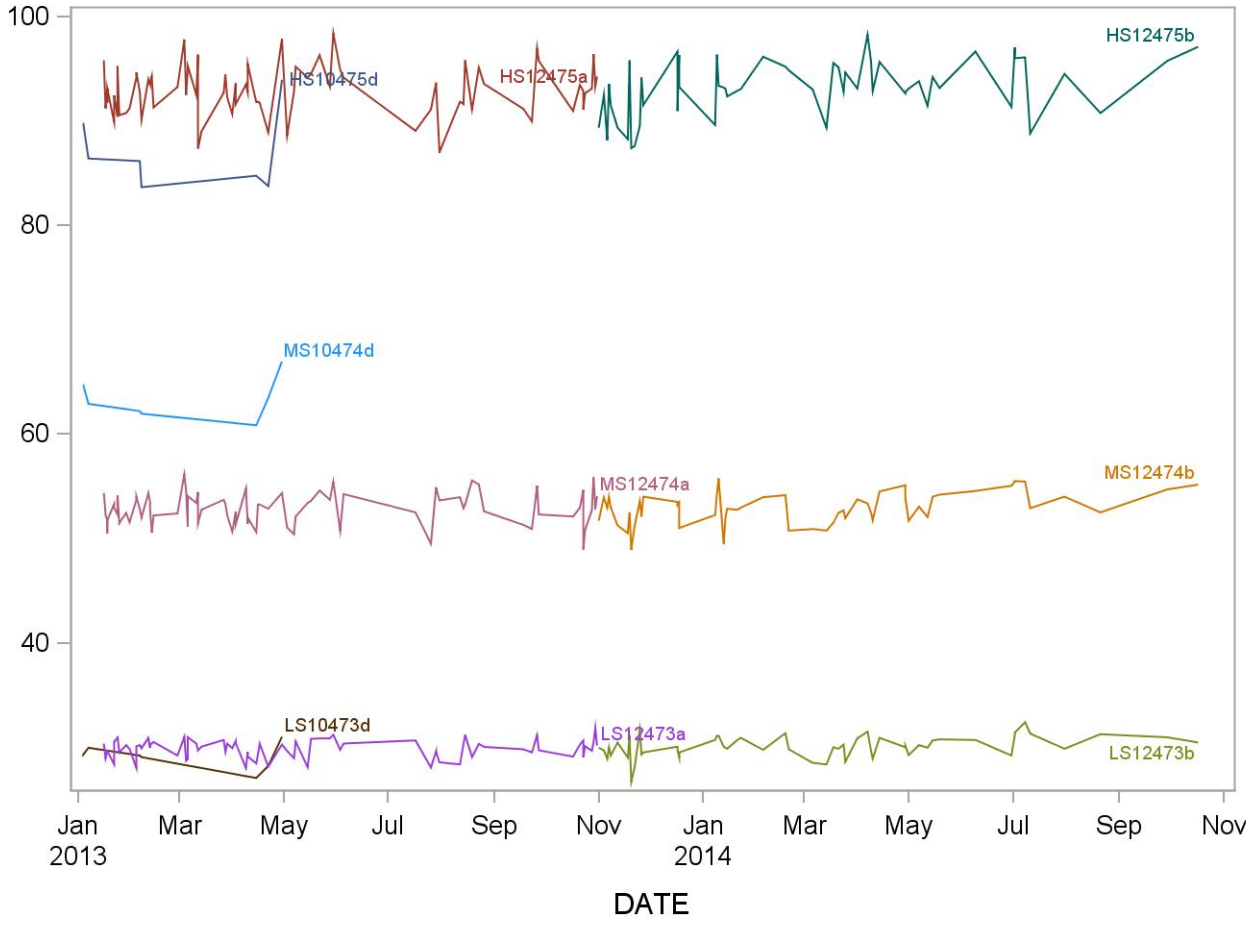
2011-2012 Summary Statistics and QC Chart for 25OHD2 (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS10475d	9	04JAN13	30APR13	39.317	1.526	3.9
LS10473d	9	04JAN13	30APR13	64.778	3.965	6.1
MS10474d	9	04JAN13	30APR13	15.033	0.725	4.8
HS12475a	82	16JAN13	31OCT13	20.998	0.928	4.4
LS12473a	81	16JAN13	31OCT13	4.904	0.389	7.9
MS12474a	82	16JAN13	31OCT13	10.439	0.504	4.8
HS12475b	61	01NOV13	17OCT14	22.049	1.053	4.8
LS12473b	61	01NOV13	17OCT14	5.013	0.419	8.4
MS12474b	61	01NOV13	17OCT14	10.866	0.564	5.2



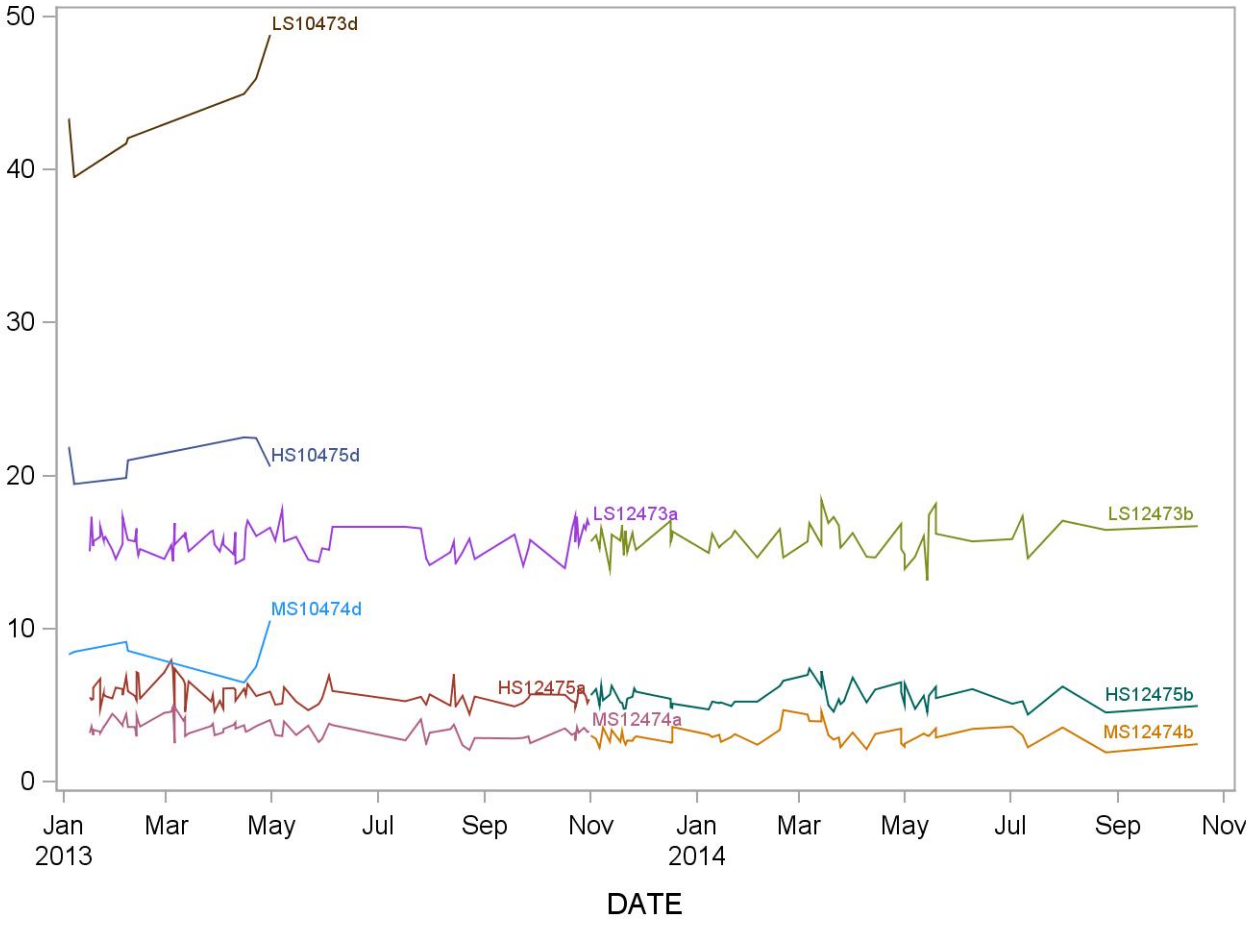
2011-2012 Summary Statistics and QC Chart for 25OHD3 (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS10475d	9	04JAN13	30APR13	87.172	3.381	3.9
LS10473d	9	04JAN13	30APR13	29.228	1.120	3.8
MS10474d	9	04JAN13	30APR13	63.400	1.821	2.9
HS12475a	84	16JAN13	31OCT13	92.894	2.392	2.6
LS12473a	83	16JAN13	31OCT13	29.857	0.890	3.0
MS12474a	84	16JAN13	31OCT13	52.890	1.487	2.8
HS12475b	56	01NOV13	17OCT14	93.162	2.705	2.9
LS12473b	56	01NOV13	17OCT14	30.075	1.025	3.4
MS12474b	56	01NOV13	17OCT14	52.988	1.551	2.9



2011-2012 Summary Statistics and QC Chart for epi-25OHD3 (nmol/L)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HS10475d	9	04JAN13	30APR13	21.006	1.221	5.8
LS10473d	9	04JAN13	30APR13	43.228	3.020	7.0
MS10474d	9	04JAN13	30APR13	8.426	1.094	13.0
HS12475a	82	16JAN13	31OCT13	5.694	0.750	13.2
LS12473a	81	16JAN13	31OCT13	15.653	0.926	5.9
MS12474a	82	16JAN13	31OCT13	3.442	0.575	16.7
HS12475b	57	01NOV13	17OCT14	5.530	0.709	12.8
LS12473b	57	01NOV13	17OCT14	15.871	1.033	6.5
MS12474b	57	01NOV13	17OCT14	2.983	0.582	19.5



Appendix A Method Figures of Merit

Accuracy

Accuracy was accessed by measuring 4 levels of serum NIST SRM 972. The mean results from 44 assays showed that the current method differed by 0%, −1% and 4% of the certified values for $25OHD_2$, $25OHD_3$, and epi-$25OHD_3$, respectively (10).

Precision

Intra-assay and inter-assay precision were assessed by measuring 3 levels of in-house serum controls. Inter-assay CV for the $25OHD_2$ (395→377), $25OHD_3$ (383→365) and epi-$25OHD_3$ (383→365) quantitation ion pairs are shown in **Table A**. Total imprecision was about 5% for $25OHD_2$ and $25OHD_3$ when concentrations were above 10 nmol/L and ≤10% for epi-$25OHD_3$ when analyte concentrations were at or above 20 nmol/L.

Table A. QC pool characterization data for 25OHD metabolites (nmol/L) from 25 assays. Three levels of QC pools were tested in duplicate in each assay; m/z transition pair is shown for each analyte.

Analyte	Pool	Mean	SD of pair means	CV
$25OHD_2$ m/z 395.3>377.3	1	38.9	1.65	4%
	2	62.7	3.87	6%
	3	13.9	0.79	6%
$25OHD_3$ m/z 383.3>365.3	1	86.1	3.70	4%
	2	28.5	1.16	4%
	3	63.7	2.45	4%
epi-$25OHD_3$ m/z 383.3>365.3	1	19.4	1.96	10%
	2	44.0	4.13	9%
	3	7.58	1.14	15%

Limits of detection

Original method limits of detection (LOD) were estimated by diluting a medium QC serum pool with PBS-4% albumin (1:30, 1:50, 1:80 and 1:100) and analyzing these diluted samples in 6 assays. A SAS program was used to estimate the standard deviation of near-blank using a modification of Taylor (12) (i.e., regression of the analytic standard deviation onto sample concentration LOD. The limits of detection (nmol/L) were calculated as follows: 3.00, 4.88, and 1.88 for $25OHD_2$, $25OHD_3$ and epi-$25OHD_3$.

Expanded method limits of detection (LOD) were estimated by diluting a medium QC serum pool with PBS-4% albumin (1:4, 1:5, 1:6, 1:10, 1:20 and 1:80) and analyzing these diluted samples in singlicate over 60 assays. A SAS program was used to estimate the standard deviation of blank and near-blank samples with a false positive and false negative probability of 5%. The current limits of detection are (nmol/L): 2.05, 2.23, and 1.64 for $25OHD_2$, $25OHD_3$ and epi-$25OHD_3$, respectively.

Appendix B Ruggedness Testing

1. **Sample Preparation Conditions – Manual vs Hamilton vs Hybrid**

a. Principle: Pipetting and mixing serum with solvents and internal standard could be problematic during the various stages of preparation. Errors may occur in delivering serum or internal standard, incomplete mixing during extraction, or errors during hexane transfer may occur. This test demonstrates the ability to use different pipetting methods at any stage of sample preparation to circumvent errors.

b. Proposal: Process samples using three different methods: 1) manually using all manual pipettes for liquid handling, dry ice bath freezing, manual pour-off of all hexane, manual reconstitution, and manual syringe filtration; 2) robotically using Hamilton STARlet for automated pipetting and mixing of serum, internal standards, and solvents, remove to rack vortexer, then back to robotically automated transfer of 1 mL hexane to well plate, plate dry-down, followed by automated reconstitution in well plate; and 3) using a hybrid method using Hamilton pipetting of serum, internal standards, and solvents, then manual completion of process. For results, see run dates of 4/5/10, 4/27/10, and 8/31/10 and summary in table below.

c. Conclusion: Alterations to the pipetting technique (manual, robotic, or hybrid method) may be done without adverse effect.

Factor	Method specifies	SRM 972 Results (nmol/L)	Ratio Results/Expected
Manual preparation only	100 µL manual pipette 75 µL pos disp pipette 0.45 µm syringe filter	SRM972 25OHD$_2$: 3.16, 5.84, 63.2, 7.54 SRM972 25OHD$_3$: 66.1, 34.7, 46.4, 77.6 SRM972 epi-25OHD$_3$: 4.99, 4.12, 2.50, 95.6	25OHD$_2$: 2.2, 1.4, 1.0, 1.3 mean=2.0 25OHD$_3$: 1.1, 1.3, 1.0, 0.9 mean=1.0 epi-25OHD$_3$: 1.4, 2.1, 0.9, 1.0 mean=1.4
Hamilton preparation only	All Robotic pipetting No syringe filters	SRM972 25OHD$_2$: 1.95, 4.62, 70.0, 5.59 SRM972 25OHD$_3$: 64.6, 29.2, 45.7, 80.1 SRM972 epi-25OHD$_3$: 3.74, 0.75, 0.75, 83.9	25OHD$_2$: 0.9, 0.9, 1.1, 0.9 mean=1.0 25OHD$_3$: 1.0, 0.9, 0.9, 0.9 mean=0.94 epi-25OHD$_3$: 1.0, 1.0, 1.1, 1.0 mean=1.0
Hybrid preparation Manual+Hamilton	Robotic pipetting 100 µL manual pipette 0.45 µm syringe filter	SRM972 25OHD$_2$: 0.97, 3.65, 68.3, 5.84 SRM972 25OHD$_3$: 49.4, 28.1, 43.7, 69.6 SRM972 epi-25OHD$_3$: 3.24, 1.50, 1.75, 93.4	25OHD$_2$: 0.7, 0.9, 1.1, 1.0 mean=0.9 25OHD$_3$: 0.8, 0.9, 0.9, 0.8 mean=0.9 epi-25OHD$_3$: 0.9, 0.8, 0.6, 1.0 mean=0.8

2. Sample Preparation Conditions – Plate Dryer vs SpeedVac

a. Principle: Following extraction, the hexane layer must be dried-off prior to final reconstitution with 69-72% methanol in 28-31% water. This drying process must be carefully carried out to ensure that the analytes remain intact in the well plate to allow maximal recovery. There are numerous techniques available for drying the hexane layer. The current method specifies using a Speedvac operated without heat at maximal vacuum to prevent the hexane from boiling. The alternative method is to use a plate dryer using either air or N_2 at controlled flow rates to achieve the drying. This test demonstrates the ability to use different drying methods.

b. Proposal: We have established the Speedvac as the preferred technique for hexane drying. Here we use the plate dryer with either house air or high purity cylinder N_2 to dry the hexane. We compare the raw analyte areas resulting from the plate dryer techniques to the Speedvac areas to assess if there is a significant loss of analyte signal. For results, see summary in table below. (Run dates = 5/25/10, 6/7/10, 6/9/10)

c. Conclusion: Use of the plate dryer with cylinder N_2 is deemed essentially equivalent to the Speedvac with <3% overall signal loss and may be used in the event the Speedvac is not available.

Factor	Method specifies	Peak Areas (Different from SpeedVac)	Overall
Plate Dryer (Air)	Dry hexane to dryness	All QC 25OHD$_2$: -10.6% All QC 25OHD$_3$: -9.8% All QC epi-25OHD$_3$: -4.2%	Overall signal reduction by 8.4%
Plate Dryer (N_2)	Dry hexane to dryness	All QC 25OHD$_2$: -2.5% All QC 25OHD$_3$: -4.6% All QC epi-25OHD$_3$: -1.3%	Overall signal reduction by 2.8%
SpeedVac	Method designated procedure to dry hexane to dryness	Reference	

Appendix C Confirmation of Peak Identity

In this analytical method, we monitor two ion pairs per reportable analyte as a means to evaluate peak identity. The ion pairs are designated 'quantifier' or 'qualifier'. For each analyte, an ion (m/z) is selected to travel to the collision cell to be fragmented. For example, for $25OHD_2$, m/z 395, which is the parent compound minus water (413-18), is fragmented into m/z 377 and m/z 209 where the former is detected as the quantifier ion and the latter is detected as the qualifier ion. Thus, 395/377 is the quantifier ion pair and 395/209 is the qualifier ion pair for $25OHD_2$. The quantifier ion pair provides the stronger signal and is used to report results. The qualifier ion pair, sometimes called the confirmation ion pair, is used to monitor the ratio of these signals for the analyte; it is usually the second strongest signal. Under standard conditions, each analyte displays a characteristic spectrum of ions that are produced by collision events in the mass spectrometer. The spectrum is characteristic for the chemical compound. Using two relatively strong signals (quantifier and qualifier), we can assess whether it is likely that the quantifier ion is misidentifying the compound of interest by the ratio of the signals, which should be consistent.

The criteria for this assessment were developed by averaging four years of valid NHANES data using peak area from the primary quantifier and secondary qualifier ions. We also estimated analyte concentrations using quantifier and qualifier ions to compare the ratio of these two concentrations, which ideally should be 1.0. We developed a scheme for not reporting data due to potential interference, based on these two rules. In each case, the peak area ratio or concentration ratio was specified to be within 30% of the expected value.

For each 25-hyrdoxyvitamin D metabolite, only those quantifier ion results greater than the limit of quantitation (LOQ) were considered when establishing the mean peak area ratios because of the inherent imprecision of results <LOQ. Similarly, only results greater than or equal to LOQ were evaluated against these rules. LOQ was not experimentally determined, but was derived by dividing the LOD by 3 and then multiplying by 10.

Peak area ratio is the ratio of the raw areas (uncorrected by internal standard) of the quantifier to the qualifier ion. Peak area ratios for each result are compared and evaluated against an expected ratio for each analyte (rule 1).

Concentrations were obtained by interpolating the relative response ratio from individual calibration curves for each ion pair. The relative response ratio is the peak area of each analyte to its internal standard in any sample, e.g., $25OHD_3$ peak area ÷ *d6*-$25OHD_3$ peak area = relative response ratio for the quantifier ion used for reporting $25OHD_3$. A concentration ratio of the qualifier ion to the quantifier ion of 1.0 is indicative of no interference by other compounds (rule 2).

Rule 1: valid results >LOQ should have a **peak area ratio** of the qualifier to the quantifier ion within ± 30% of the average peak area ratio of the qualifier to the quantifier ion calculated from NHANES 2007-2010. This criterion was selected from the literature as an appropriate amount of variability beyond which interference could be expected [1, 2].

Rule 2: valid results >LOQ should have **concentrations** calculated using the qualifier ion and quantifier ion that ratio (qualifier/quantifier) to within ± 30% of 1.0. The use of this criterion was implemented in our lab as quantitative measure of interference because all secondary qualifier ions are quantified in the same manner as the primary quantifier ions via multi-point calibration curves, hence the concentration ratios outside of unity provides suggestive evidence of interference. These two rules were applied to individual samples in the following way:

Rule #1	Rule #2	Status
PASS	PASS	Reportable
FAIL	PASS	Use judgment
PASS	FAIL	Use judgment
FAIL	FAIL	Non-reportable

Summary of findings: confirmation of peak identity applied to NHANES 2007-2010 results*

Summary	**25OHD$_2$**	**25OHD$_3$**	**Epi-25OHD$_3$**
Quantifier m/z pair	395.3/377.3	383.3/365.3	383.3/365.3
Qualifier m/z pair	395.3/209.1	383.3/105.1	383.3/105.1
Rule #1: m/z pair peak area ratio	0.54 ± 30%	0.37 ± 30%	0.37 ± 30%
Rule #2: m/z pair concentration ratio	1.0 ± 30%	1.0 ± 30%	1.0 ± 30%
No. results >LOQ (total results)	1,513 (16,826)	16,813 (16,826)	3,049 (16,826)
Reportable results ,% (n)			
#1 PASS/ #2 PASS	99% (1,492)	98% (16,561)	84% (2,560)
#1 FAIL/ #2 PASS	1% (17)	2% (252)	9% (261)
#1 PASS/ #2 FAIL	<1% (2)	0% (0)	<1% (2)
Non-reportable results, % (n)			
#1 PASS/ #2 FAIL	0% (0)	0% (0)	2% (55)
#1 FAIL/ #2 FAIL	<1% (2)	0% (0)	6% (171)
Total non-reportable results, % (n)	**<1% (2)**	**0% (0)**	**7% (226)**

*****includes NCEH and NCHS bench and blind QC

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