Laboratory Procedure Manual

Analyte: **25-Hydroxyvitamin D_3, C3-epimer-25-Hydroxyvitamin D_3, and 25-Hydroxyvitamin D_2**

Matrix: **Serum**

Method: **High Performance Liquid Chromatography-Tandem Mass Spectrometry**

Method No: 4027.07

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as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
National Center for Environmental Health (NCEH)

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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.

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

Lab Number	Analyte	SAS Label (and SI units)
VID_I	LBXVIDMS	Total Vitamin 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 Clinical Relevance and Principle

A. 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 25(OH)D_3 isomers allowing for the specific quantitation of the major biological forms (in persons ≥ 1y) of $25OHD_3$ and $25OHD_2$. Valid $25OHD_3$ and $25OHD_2$ results are summed to total 25-hydroxyvitamin D (25OHD). It should be noted that 25-hydroxyvitamin D_2 also has 3β- and 3α- isomers, which this method has the ability to separate, but due to the uncommon occurrence of the 3α form, these data are not collected.

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]**.

B. Test Principle

The test principle for the CDC method utilizes high performance liquid chromatography-tandem mass spectrometry (HPLC-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 three pentaflurophenyl (PFP) columns (Thermo Scientific Hypersil GOLD PFP 2.1 x 100 mm, 1.9 µm particle size column, Phenomenex Kinetex PFP 2.1 x 100 mm, 1.7 µm, or Sigma-Aldrich Ascentis Express F5, 2.1 x 150 mm, 2.7 µm). Mobile phase composition for optimized chromatography varies slightly for the three 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, and the needle wash should match that used for the mobile phase.

Serum samples are first treated by the addition of a 67% methanol solution containing three internal standards (IS) 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 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 unknown 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. We recommend 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, under a chemical fume hood.

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

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 or automated integration 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 or STARLIMS database for review of the data, statistical evaluation of QC/QA data, and approval of the results. See **4027 SOP VID Computerization and Data System Management** and **4027 SOP VID Starlims Data Review** for a step-by-step description of data transfer, review, and approval.

(C) For NHANES, data are transmitted electronically on a regular basis (approximately weekly for certain 3-week turnaround analytes) or at the end of a survey. 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, NCHS generally makes arrangements for abnormal report notifications by the NCHS Survey Physician.

(D) The instrument raw and results files (including all patient, QC, and calibration data) on instrument computer hard drives are backed up to the CDC network 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) A fasting specimen 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 500 µL is required for the assay to have sufficient volume to permit adequate automated pipetting volume and repeat analysis, if necessary.

(D) Specimens may be stored in glass or plastic vials, if 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.gov\project\CCEHIP_NCEH_DLS_NBB_LABS\CLIA\DLS Policies and Procedures Manual). The protocol discusses collection and transport of specimens and the special equipment required. In general, serum should be transported and stored at no higher than -20˚C. Generally, specimens thawed and refrozen less than five times are not compromised. If there are multiple tests 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; avoid cross-contamination.

(I) There are no known rejection criteria that would necessitate rejecting a specimen for 25OHD analysis. However, specimens preserved with sodium citrate will require that the volume of the citrate preservative be provided so that a mathematical correction may be applied to correct for the dilution created using citrate.

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

Though each reagent preparation step specifies a total volume of reagent to be prepared, these directions may be scaled up or down to prepare larger of smaller quantities if desired.

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.

Most reagent preparations are conducted in non-volumetric glassware. Use class A volumetric glassware where a volumetric flask is specified.

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. While not required, it is ideal to place the mobile phase bottle in a sonicator to remove excess gas, for at least 30 minutes. This solution is stable at room temperature, so prepare as needed.

2) HPLC Mobile Phase – 100% Methanol

Methanol is added to a clean 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. While not required, it is ideal to place the needle wash bottle in a sonicator to remove excess gas, for at least 30 minutes. This solution is stable at room temperature, so prepare as needed.

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

Use Sigma catalog number P-3813 or a comparable product; 1 packet is dissolved in 1 liter of water. This solution is stable at room temperature and is used for the matrix blank (4% albumin in 0.01M PBS).

5) 4% Albumin in 0.01 M PBS

Weight out 2 grams of albumin and dissolve in 50 mL of 0.01 M phosphate buffered saline. Vortex or stir it on a magnetic stirrer until it totally dissolved. This material is the extraction blank. Prepare fresh once every two weeks and store it in a refrigerator at 4 ˚C or prepare a large batch as needed, aliquot 500 µL into 1.8-mL cryovials and store in -70 ˚C.

6) Mass Spectrometer Equilibration Test Solution

Prepare the equilibration test solution in 20-mL scintillation vials. Add the appropriate volume of deuterated and non-deuterated $25OHD_3$, $25OHD_2$, epi-$25OHD_3$ stock into the 69-72% MeOH/water solution to make a target concentration of approximately 90 nmol/L for each analyte. Vortex the solution thoroughly. The concentration can be adjusted as needed. The test solution is stored in the refrigerator at 4 ˚C and is prepared as needed.

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

a) 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 Class A volumetric flask). Bring to volume with ethanol, and mix.

b) 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 Class A volumetric flask). Bring to volume with ethanol, and mix.

c) 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 Class A volumetric flask). Bring to volume with ethanol, and mix.

d) Using a calibrated UV/vis spectrophotometer, measure the absorbance (AU) of each stock solution at 264 nm 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: **$AU = c*\varepsilon 264*\ell$**

1. AU is defined as absorbance units
2. c is the concentration
3. ε264 is the extinction coefficient of all three analytes at 264nm = 18,200 L/mol*cm
4. l is the path length, which in this case is 1 cm

e) 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 * $1e^9$ (or $c_{nmol}/L = AU*54{,}945$).

f) For ng/mL, on the other hand, the extinction coefficients must be adjusted by the atomic mass (AMU) of each metabolite; AMU=412.7 g/mol for 25OHD$_2$; AMU=400.6 g/mol for 25OHD$_3$ and 3-epi-25OHD$_3$

g) The conversion for ε_{264} is (18,200L/mol*cm ÷ AMU g/mol) ÷ $1e^5$. Hence:

1. ε_{264} (25OHD$_2$) = $4.40998e^{-5}$ mL/ng*cm
2. ε_{264} (25OHD$_3$ & 3-epi-25OHD$_3$) = $4.54319e^{-5}$ mL/ng*cm

2) Calibration Solutions

Calibration solutions are prepared by mixing the appropriate volume of each stock solution with 70% methanol/water or a matrix such as 4% albumin or serum in Class A volumetric flasks. A typical calibration preparation process for calibrators is to prepare an intermediate calibration solution containing all three analytes from which all subsequent calibrators are made via the indicated dilutions (**Table 1a** and **Table 1b**) with either solvent (70% methanol/water), 4% albumin in PBS, or serum. Each lot will vary in concentration depending upon the concentration of the intermediate solution and the levels desired for each calibrator. Each calibrator is thoroughly vortexed and stored at -70 °C until use. The number of calibration points prepared can vary from 6 calibrators up to 8 calibrators. These extra calibrators may be made to enhance a particular region of the calibration,

but the addition of additional calibrators also reduce the number of unknowns that can be analyzed in a run.

Table 1a: Summary of typical calibrator preparation (solvent or albumin-PBS matrix)

Calibrator ID	Volumes blended for each calibrator (µL)					*Final concentration (nmol/L)		
	volume 25OHD$_2$ stock	volume 25OHD$_3$ stock	volume epi-25OHD$_3$ stock	Intermediate Stock volume	matrix volume	25OHD$_2$	25OHD$_3$	epi-25OHD$_3$
Stock concentration	-	-	-	-	-	38,181.3	42,368.1	51,254.6
Intermediate solution #1	838	1,770	400	-	**46,992	639.9	1,499.8	410.0
Calibrators below made from Intermediate Solution #1 (µL)								
Cal 8				5,000	20,000	128	300	82.0
Cal 7	-	-	-	10,000	90,000	64.0	150	41.0
Cal 6	-	-	-	7,000	93,000	44.8	105	28.7
Cal 5	-	-	-	5,500	94,500	32.0	82.5	22.6
Cal 4	-	-	-	3,500	96,500	22.4	52.5	14.4
Cal 3	-	-	-	2,000	98,000	12.8	30.0	8.20
Cal 2	-	-	-	1,000	99,000	6.40	15.0	4.10
Cal 1	-	-	-	420	99,580	2.69	6.30	1.72

*Final concentrations of calibrators shown are the theoretical target values. Calibrator values are re-assigned after preparation based upon harmonization with SRM materials and/or results from DLS method 4029 (reference method).

**Intermediate solution #1 is prepared in 70% methanol/water regardless of the calibrator matrix used for cal1 through cal8.

Table 1b: Summary of typical calibrator preparation (serum matrix)

Calibrator ID	Volumes blended for each calibrator (µL)							*Final concentration (nmol/L)		
	volume 25OHD$_2$ stock	volume 25OHD$_3$ stock	volume epi-25OHD$_3$ stock	calibrator volume	matrix volume (100% ethanol)	matrix volume (serum)	matrix volume (4% albumin in PBS)	25OHD$_2$	25OHD$_3$	epi-25OHD$_3$
Stock concentration	-	-	-	-		-	-	38,181.3	42,368.1	51,254.6
Intermediate solution #2	500	833	250	-	917	-	-	7,636.3	14,122.7	5,125.5
Intermediate solution #1	80.0	53.3	42.1	-	625	-	-	3,818.1	2,824.5	2,697.6
Serum Concentration (Baseline)								0.60	25.1	0.50
	Calibrators made from stock (µL)									
Cal 8	320	650	140	-	-	98,890	-	123	300	0.64
	Calibrators made from Intermediate Solution #2 (µL)**									
Cal 7	-	-	-	850	-	99,150	-	65.5	145	44.1
Cal 6	-	-	-	600	-	99,400	-	46.4	110	31.2
Cal 5	-	-	-	400	-	99,600	-	31.1	81.5	21.0
Cal 4	-	-	-	260	-	99,740	-	20.5	61.8	13.8
	Calibrators made from Intermediate Solution #1 (µL)**									
Cal 3	-	-	-	280	-	99,720	-	11.3	32.9	8.05
Cal 2	-	-	-	150	-	49,925	49,925	6.05	16.8	4.29
Cal 1	-	-	-	60	-	19,988	89,952	2.19	6.10	1.56

*Final concentrations of calibrators shown are the target values. Calibrator values are re-assigned after preparation based upon harmonization with SRM materials and/or results from DLS method 4029 (reference method).

**The concentrations of intermediate solutions and volume added to each calibrator level is adjusted based on the baseline concentration of matrix (serum).

3) Internal Standard Solutions (Stock and Working)

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

a) *d6*-25OHD$_3$: 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.

b) *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.

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 0.05 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 67% methanol in water solution as diluent to obtain a final concentration of 75 nmol/L *d6*-25OHD$_3$,

20 nmol/L *d3*-25OHD$_2$, and 15 nmol/L *d3*-3-epi-25OHD$_3$. Concentrations may be adjusted as needed.

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$_2$, 30-86 nmol/L for 25OHD$_3$ and 2-20 nmol/L for 3-epi-25OHD$_3$.

To prepare pools, first prescreen units for 25OHD$_2$, 25OHD$_3$ and 3-epi-25(OH)D$_3$ 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 clean technique under a laminar-flow hood, dispense the continuously mixed serum in 300-500-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.

At least 6 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

With some exceptions, a material listed herein may be substituted with an equivalent product from a different manufacturer if it meets or exceeds the specifications of the product listed. In the case of standards, internal standards, chemicals, and reagents, the chemical and/or isotopic purity of the substitute must meet or exceed that of the listed product. In the case of the HPLC column and guard cartridges, equivalent performance must be demonstrated experimentally in accordance with the *DLS Policies and Procedures*.

(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) Ascentis Express F5 2.1 x 150 mm: 2.7 µm particle size column (Sigma-Aldrich, St. Louis, MO)

(4) KrudKatcher Ultra Inline HPLC filter, 0.5 µm depth x 0.004 in ID (Phenomenex, Torrance CA)

(5) 2.1 mm ID x 2 µm inline filter (Thermo Scientific, Belefonte, PA)

(6) 13 x 100 mm Disposable glass culture tubes (Corning Glassworks, Corning, NY)

(7) 5¾" Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)

(8) Solvent filters, 0.45 µm pore size (Millipore Corp, Medford, MA)

(9) N-Dex nitrile examination gloves (Best Manufacturing Corp, Menlo, GA)

(10) 0.45 µm Syringe tip PVDF hydrophilic filter (4 mm diameter) (obtained from various sources)

(11) Plastic tuberculin syringes (obtained from various sources)

(12) 1.8-mL Polypropylene cryovials (Nalgene Company, Rochester, NY)

(13) Various glass beakers, volumetric flasks, graduated cylinders and bottles (class A glassware)

(14) Methanol, HPLC grade (Tedia, Fairfield, OH or Honeywell, Morris Plains, NJ)

(15) Hexane, HPLC grade (Fisher Scientific, Pittsburg, PA)

(16) Ethanol, HPLC grade (obtained from various sources)

(17) Albumin from bovine serum (Sigma, St. Louis, MO)

(18) 25-Hydroxyvitamin D_3 (USP, Rockville, MD; Sigma, St. Louis, MO)

(19) 25-Hydroxyvitamin D_2 (Isosciences, King of Prussia, PA; Sigma, St. Louis, MO)

(20) 3-Epi-25-Hydroxyvitamin D_3 (Isosciences, King of Prussia , PA)

(21) 26,27-Hexadeuterium-25-hydroxyvitamin D_3 (Medical Isotopes Inc, Pelham, NH)

(22) 6,19-Trideuterium-25-hydroxyvitamin D_2 (Isosciences, King of Prussia, Pa.)

(23) 6,19-Trideuterium-3-Epi-25-hydroxyvitamin D_3 (Medical Isotopes Inc, Pelham, NH)

(24) Rainin pipette tips, 200- and 1000-µL (Rainin Instrument, LLC, Woburn, MA)

(25) Gilson Microman positive displacement pipette tips, 100 µL and 250 µL (Gilson, Villiers-le, France)

(26) Parafilm, 4-inch wide roll (any vendor)

(27) 96-Cell round bottom well plates, 1.2-mL (Fisher Scientific, Pittsburg, PA)

(28) Preslit silicone plate seals 8.6 mm (Fisher Scientific, Pittsburg, PA)

(29) Hamilton Robotic liquid handler 300-µL and 1000-µL tips (Hamilton, Reno, NV)

E. Instrumentation

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with an equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of analytical instrumentation (e.g., HPLC components, tandem quadrupole mass spectrometer) equivalent performance must be demonstrated experimentally in accordance with the *DLS Policies and Procedures* when multiple analysis systems are used in parallel, even if they are of the exact same type.

(1) Thermo Vantage mass spectrometer, with Xcalibur software (ThermoElectron Corp, West Palm Beach, FL)

(2) Thermo Accela UHPLC system (ThermoElectron Corp)

(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)

(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)

(10) Magnetic stirrer (Fisher Scientific Co., Fairlawn, NJ)

(11) Mettler Toledo analytical balance, model XP205, XPE205 and ML203T (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)

7. Calibration and Calibration Verification Procedures

A. Instrument Calibration and Calibration Verification

1) Thermo LC-MS/MS

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 Thermo LCMSMS Systems** 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 Thermo LCMSMS Systems** for detailed instructions.

2) Hamilton Microlab Starlet

Twice a year, a Hamilton Service Engineer performs a preventative maintenance including volume verification. Users can check instrument performance through gravimetric volume verification of the various steps of the method using volume field verification kit from Hamilton. Users can also run the optional daily and weekly maintenance programs as additional performance checks.

B. Assay Calibration and Calibration Verification

At the beginning of each run, six or more mixed calibrators (containing $25OHD_3$, epi-$25OHD_3$, and $25OHD_2$) with concentrations ranging from about 2 to 300 nmol/L are prepared as described in section 6.b. of this document. Four calibration preparation techniques have been validated for use by this method using either direct injection or extraction of the calibrators. The method currently uses the serum-based calibration; however, any one of the following techniques are appropriate if required:

a) solvent-based calibrators may be directly injected

b) solvent-based calibrators may be extracted with the addition of either water or 4% albumin in PBS

c) 4% albumin in PBS-based calibrators must be extracted

d) serum-based calibrators must be extracted (current calibration method)

The use of direct injection is simple and straightforward. However, if precision problems are observed due to environmental conditions, such as evaporation, then the use of calibration extraction is preferred. The alternate preparation processes of the calibration materials are described in the subsequent sections below. Calibration 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*-$25OHD_2$. Routinely, 12- to 16-point linear curves (6-8 points from the front and 6-8 points from the back of the run), 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 prepared at the same time as the unknowns and if extracted, are carried through the sample preparation procedure. Curves are weighted as follows: $25OHD_3$ ($1/x$); $25OHD_2$ ($1/x^2$); epi-$25OHD_3$ ($1/x^2$).

(1) Solvent based calibration, no addition of matrix:

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, hence a solvent-based calibration was instituted in 2013. The solvent-based calibration was compared to the original matrix-based calibration (4% albumin in PBS) and found to be comparable and produced satisfactory results on NIST reference materials. Prior to that, the 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. A <5% difference in the average calibration curve slopes was observed between calibrators containing serum and albumin-PBS calibrations for all three analytes. The differences observed were of a similar magnitude to slope variability observed within and between individual calibration curves of a particular matrix. A 100-µL aliquot calibrator is mixed with a 75-µL of internal standard solution. Peak area ratios as described above are used. Curves are similarly weighted.

(2) Extracted calibration, 4% albumin matrix, serum matrix, or water addition:

While the method does not *require* a carrier protein for calibration, it has been observed that day-to-day imprecision and internal standard variations are sometimes high, and this is likely due to room temperature changes (within room or between rooms) during the day. We have shown that taking the calibration materials through the extraction process with the addition of a matrix or DI water often reduces these fluctuations.

To extract calibrators, a 100-µL aliquot calibrator is mixed with a 75-µL of internal standard solution then 100-µL of 4% albumin in PBS or DI water is added. The resulting solution is carried thru the full extraction process as described in section 8a and 8b. Peak area ratios as described above are used. Curves are similarly weighted.

If the test system calibration procedure includes:

a) three or more levels of calibration materials

b) a low point near the LOD, mid, and high values

c) and is performed at least once every six months, then the requirement for calibration verification is also met.

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) must be demonstrated in accordance with CDC DLS Policies and Procedures Manual when multiple analysis systems are used in parallel, even if they are of the exact same type. The comparisons involve analyzing several samples on each of the instruments and assessing the resulting Pearson correlation coefficients. Details about these procedures can be found in **4027 SOP Instrument Comparison & System Verification**.

The CDC laboratory participates in multiple proficiency testing programs for 25OHD. The primary one is sponsored by DEQAS (Vitamin D External Quality Assessment Scheme); others are sponsored by National Institutes of Standards and Technology (NIST, Gaithersburg, MD), CDC's Vitamin D Standardization and Certification program (VDSCP) and CAP. Every three months, 5 specimens are sent by DEQAS (20 specimens per year). This is the primary PT program, but the CDC laboratory participates in alternative PT programs. For general information on the handling, analysis, review, and reporting of proficiency testing materials see **NBB_SOP Proficiency Testing Procedure**.

In addition, NIST makes available 4 levels of Standard Reference Materials (SRM 972a) for serum $25OHD_3$, 3-epi-$25OHD_3$, and $25OHD_2$, containing certified and reference values; these materials are tested at least four times a year. NIST also provides certified ethanol solutions SRM 2972a for $25OHD_3$, 3-epi-$25OHD_3$ and $25OHD_2$ and these are used to verify our stock standard solution concentrations as needed. Reference Method Procedures (JCTLM C12RMP2 and C12RMP3) also provide reference values for numerous samples throughout the year for additional verification.

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 Hamilton Liquid Handlers** for detailed method specific instructions.

(4) Extraction Phase:

a) Step 1: Transfer 100 µL of QC, calibrators, or patient serum sample to thin walled 13 x 100 mm borosilicate tubes.

b) Step 1b (If extracting solvent-based calibrators): Transfer 100 µL of 4% albumin in PBS or DI water to each calibrator tube and allow robotic mixing.

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

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

e) 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.

f) 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.

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

(5) Evaporation and Reconstitution Phase:

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

b) 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 for 45 mins at room temperature. If using a TurboVap, dry under nitrogen gas at the flow rate of 50-60 standard cubic feet per hour (SCFH) for 30 mins at room temperature.

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

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

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

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

B. Sample Preparation (manual preparation)

(1) Prepare an ethanol/dry ice bath (temperatures -70 °C).

(2) For manual preparation see # 1, 2, and 4 in above section 8 A. Use a manual pipette to transfer samples and an automated pipette, such as a Digiflex, to transfer the hexane.

(3) Place the tubes into the ethanol/dry ice bath ensuring that the liquid layer is submerged into the bath. Allow to freeze for 25 minutes or more.

(4) Leaving the tubes in the ethanol bath, remove one tube at a time and pour the hexane (upper) layer from the 13 x 100 mm tube into a pre-labeled 12 x 75 mm tube until all tubes have been transferred. Discard the 13 x 100 mm tube containing the frozen aqueous layer into a biohazard discard pan.

(5) Load the tubes containing the hexane layer into an unheated Speedvac to evaporate the hexane to dryness. Follow the Speedvac manufacture's instruction manual for specific steps for drying hexane.

(6) To the dry tubes add 300 µL 69-72% methanol (column-dependent) using a manual or an automated pipette, such as a Digiflex.

(7) Take the reconsitituted tubes to a multi-plate shaker and shake for 1 minute at 2,000 setting.

(8) Using a 1-mL disposable syringe, completely draw up the solution from each tube, attach a 0.45-µm syringe-driven filter to the end of the syringe, and dispense the contents into the designated well in a pre-labeled 96-well plate (suggest preparing a worksheet prior to analysis to help ensure that every specimen is properly identified and transferred).

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

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

C. LC-MS/MS Analysis

(1) Columns are held at 28 °C under isocratic conditions. A variety of columns may be used for this assay and are not limited to the ones shown in the materials list. As columns become commercially available, they may be tested to ensure appropriate elution retention times, adequate separation, and acceptable back pressures. If the columns perform well, they may be employed for this method. Currently, the analytes are eluted from one of three analytical columns:

Mobile Phase Composition	Flow Rate	Column
~69% methanol:31%water	~400 µL/min	Thermo Hypersil
~72% methanol:28%water	~400 µL/min	Phenomenex Kinetex
~72% methanol:28%water	~350 µL/min	Ascentis Express F5

(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 (25OHD2-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*-$25OHD_2$** *m/z* 398.3→380.3. The elution order of the analytes is $25OHD_3$, epi-$25OHD_3$, 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.

(4) 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 was initially monitored. Currently area of the qualitative ion ÷ quantitative 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**.

(5) The MS instrument settings are generally as follows: Currents: corona current =7.0 µA; Voltages: Collision energy = 16V, Declustering voltage = -8 V, 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.

(6) A portion (50 μL) of the extract 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.

D. Instrument Preparation

(1) HPLC Preparation

a) Refer to **4027 SOP Thermo LCMSMS Systems** for detailed instructions

b) Mobile phase solvents: Line #1: 69-72% methanol in water, and Line #2: 100% methanol

c) Needle wash solution: 69%-72% methanol in water

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

Refer to **4027 SOP Thermo LCMSMS Systems** for detailed instructions on preparing the instrument for analysis, shutdown and restart procedures, and cleaning procedures.

E. Run Samples on the LC-MS/MS

(1) See **4027 SOP Thermo LCMSMS Systems** 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.

F. Quantitation

(1) Refer to **4027 SOP Thermo LCMSMS Systems** for detailed instructions for using Thermo XCalibur software or **4027 VID Starlims Data Review** for Ascent Indigo Automated Integration Software to perform integrations and quantitation.

(2) Using either method, export the run to Excel, then import to the laboratory information management system database (NBB_DB, NBB_LIMS or STARLIMS) for review

G. Data Review

Refer to **4027 SOP Computerization and & Data System Management** for detailed information on data handling using the LIMS and **4027 VID Starlims Data Review** for using STARLIMS.

Calculate total 25OHD as the sum of $25OHD_3$ and $25OHD_2$, not including C3-epi-$25OHD_3$. The epimer is chromatographically resolved from $25OHD_3$ to avoid misclassification bias. Total 25OHD is not reported if the laboratory is unable to obtain a valid result for either $25OHD_3$ or $25OHD_2$.

Use an imputed value for $25OHD_2$ when it is <LOD (2.05 nmol/L), which is the case for about 80% of NHANES samples. The imputed value is the LOD divided by the square root of 2, which is 1.45 nmol/L for $25OHD_2$.

Report an imputed value for C3-epi-25OHD$_3$ when it is <LOD (1.64 nmol/L). The imputed value is 1.16 nmol/L for C3-epi-25OHD$_3$.

Check calibration curves for each analyte. Coefficients of determination should be $R^2 > 0.99$.

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, 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 in the CDC network and uploaded into STARLIMS. The LIMS is also backed-up regularly.

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

H. 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 Thermo LCMSMS Systems 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 Thermo LCMSMS Systems 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.

I. CDC Modifications

This method was published in 2011 **[8]**. This document represents the seventh version of the official method for the CDC lab.

9. Reportable Range of Results (AMR – Analytical Measurement Range)

The method described here is designed to detect serum 25OHD$_2$ and 25OHD$_3$ isomers at values from approximately 2-300 nmol/L. When 25OHD$_3$ values are <12.5 nmol/L, which was at the 10th percentile level observed and reported in the National Report on Biochemical Indicators of Diet and Nutrition in the U. S.

Population 1999-2002 **[9]**, the results are verified by re-analysis. There is no threshold level for repeats for 25OHD2 or epi-25OHD$_3$. Samples with concentrations greater than the highest calibrator are either diluted with 4% albumin in PBS and confirmed through repeat testing using the routine calibration curve or reanalyzed without dilution using the expanded calibration curve. The difference between retest values should be within acceptable limits. Otherwise, another repeat needs to be done. There is no known maximum acceptable dilution. Dilutions should be conducted in accordance to *DLS Policies and Procedures* that do not violate minimum volume requirements or serial dilutions beyond three dilution transfers.

The reportable ranges of serum concentrations are as follows:

25OHD$_3$	2.23 - ~300 nmol/L
3-epi-25OHD$_3$	1.64 - ~80.0 nmol/L
25OHD$_2$	2.05 - ~130 nmol/L

10. Quality Control (QC) Procedures

A. Blind Quality Controls

Blind QC specimens are prepared using serum pools that emulate low and high levels 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 like those found in patient samples.

B. Bench Quality Controls

Bench QC specimens are prepared generally 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 initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated periodically.

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”:

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

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

1. 1 3S Rule—Run result is outside a 3 S_i limit or
2. 2 2S Rule—Two or more of the three run results are outside the same 2 S_i limit
3. 10 X-bar Rule—Current and previous nine run results are on the same side of the characterization mean or
4. R 4S Rule—Two consecutive standardized run results differ by more than 4 S_i

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

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

1. 1 3S Rule—Run mean is outside a $3S_m$ limit or
2. 2 2S Rule—Two or more of the three run means are outside the same $2S_m$ limit or
3. 10 X-bar Rule—Current and previous nine run means are on the same side of the characterization mean

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

1. Outlier—One individual result is beyond the characterization mean ± $4S_i$ or
2. R 4S Rule—Two or more of the within-run ranges in the same run exceed $4S_w$ (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 **[10]** 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 and STARLIMS 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.

C. Sample QC Criteria

Each individual sample result is checked against established sample QC criteria limits to assure data quality. The method uses the following sample QC criteria:

- Relative retention time (retention time quantitation ion/retention time ISTD)
- Confirmation ion ratio (confirmation ion area/quantitation ion area)
- Concentration ratio (confirmation ion concentration/quantitation ion concentration)
- Percent difference of Individual ISTD area from within-run average
- Visual inspection of C3-epimer-25 hydroxyvitamin D_3 interference peaks (As needed, comment code 98 is applied to the result and the sample is submitted for repeat analysis.)

For details, see **4027 VID Starlims Data Review**.

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

(A) Look for sample preparation errors, e.g., added internal standard, specimen, etc.

(B) Check to make sure that the hardware is functioning properly. Check for leaks and clogs on the UHPLC or the robotic liquid handler.

(C) Check the calibrations of the pipettes and robotic liquid handler.

(D) Check for proper gas flow from the nitrogen generator.

(E) Check the autosampler for evidence of correct sample injections. Check statistical repeatability of multiple injections.

(F) Check column for adequate separation.

(G) Run standards to see if the molecular ion is detected.

(H) Determine if the mass spectrometer sensitivity is adequate by evaluation of ion counts in the equilibration solution.

(I) Make sure the mass spectrometer calibrations are proper. Run 1,3,6 polytyrosine solution to check the instrument calibration.

(J) 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.

(K) 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 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)

From NHANES 2007-2010, the 2.5th-97.5th percentile of 25-hydroxyvitamin D levels in the population over 1 year was 23.5 – 124 nmol/L; arithmetic mean was 68.0 nmol/L **[11]**.

Table 2 shows the 2011 Institute of Medicine (IOM) determination of the health status associated with various serum concentrations of total 25-hydroxyvitamin D **[12]**. Levels less than 30 nmol/L may be associated with increased risk of deficiency.

Several factors such as season, race (skin darkness), latitude, sun protection behaviors, 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 **[13,14]**. 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: Serum 25-hydroxyvitamin D (25OHD) 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")

Any NHANES samples with 25-hydroxyvitamin D <30 nmol/L may represent a risk for vitamin D deficiency, but at this time, low 25OHD is not considered a critical call result. However, for smaller, non-NHANES studies, abnormal values, such as levels below 30 nmol/L, may be identified to the study principal investigator, depending on individual study arrangements. Emails sent concerning abnormal results are maintained by the supervisor for the duration of the study. Most of these studies are epidemiological in nature.

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 (typically at ≤-70 °C) as soon as possible.

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

There are no suitable alternative methods for the analysis of epi-25OHD$_3$ in the Nutritional Biomarkers laboratory. 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 °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

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. Residual serum from these analyses for non-NHANES studies are retained for at least 1 year after results have been reported and may then be returned or discarded at the request of the principal investigator. Very little residual material will be available after NHANES analyses are completed; however, residual serum is retained for at least 2 years after results have been publicly released. At that point, samples with sufficient volume (>0.2 mL) are returned to NHANES, and samples with insufficient volume may be 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 IDs are entered into a database and the specimens are stored in a freezer at -70 °C. The Sample ID is read from the vial by a barcode reader used to prepare the electronic specimen table for the analytical system. When analyses are completed, result files are loaded into the database. The analyst is responsible for keeping records of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies. In general, these are documented using codes in the LIMS.

19. Method Performance Documentation

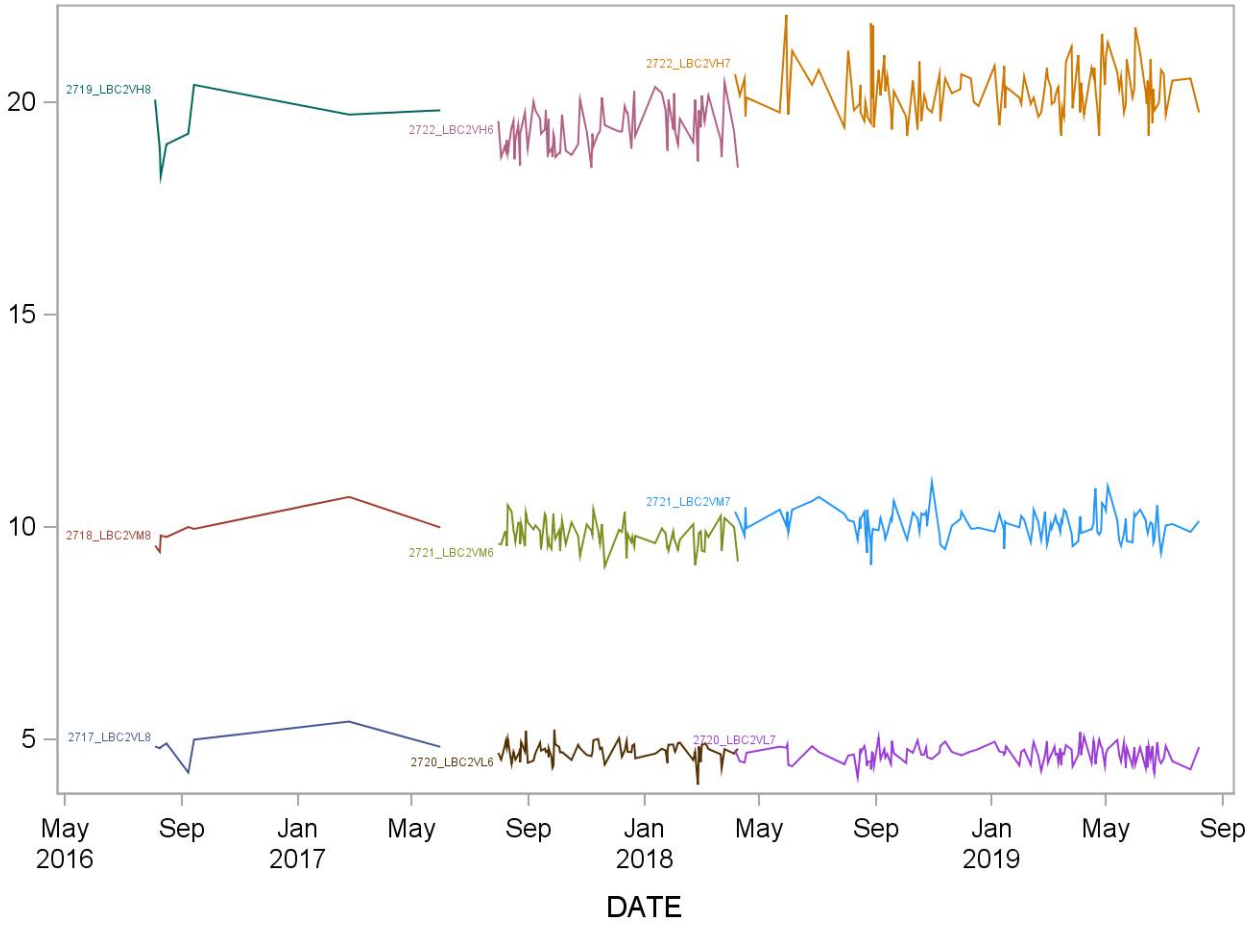
Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in **Appendix A** of this method documentation. **The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.**Method Performance Documentation

20. Summary Statistics and QC Chart

Please see following pages.

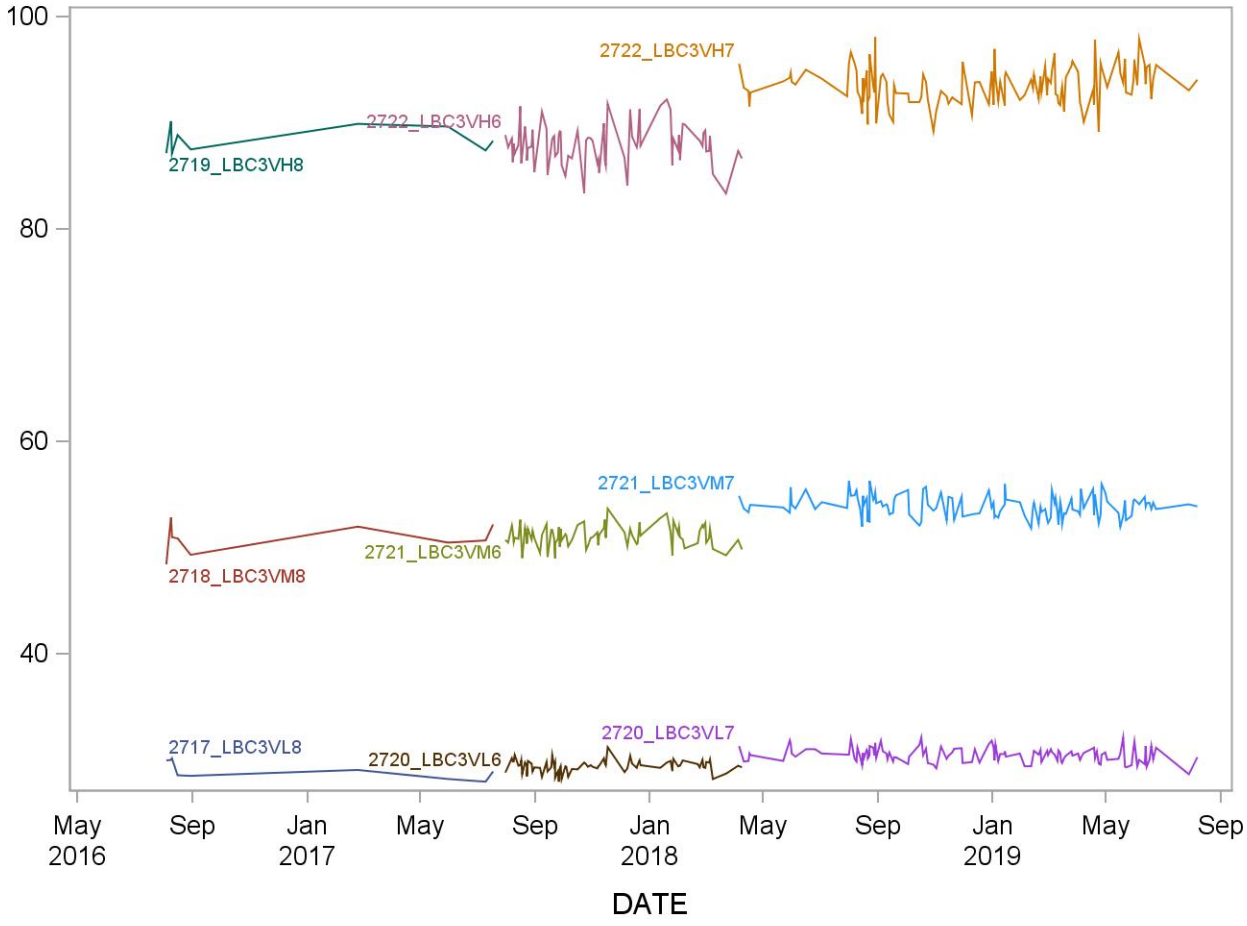
2015-2016 Summary Statistics and QC Chart
LBXVD2 (25OHD2 (nmol/L))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2719_LBC2VH8	8	04AUG16	31MAY17	19.419	0.699	3.6
2717_LBC2VL8	8	04AUG16	31MAY17	4.841	0.328	6.8
2718_LBC2VM8	8	04AUG16	31MAY17	9.890	0.388	3.9
2722_LBC2VH6	80	31JUL17	09APR18	19.336	0.502	2.6
2720_LBC2VL6	80	31JUL17	09APR18	4.711	0.204	4.3
2721_LBC2VM6	80	31JUL17	09APR18	9.796	0.312	3.2
2722_LBC2VH7	126	06APR18	07AUG19	20.254	0.605	3.0
2720_LBC2VL7	126	06APR18	07AUG19	4.646	0.206	4.4
2721_LBC2VM7	125	06APR18	07AUG19	10.043	0.330	3.3



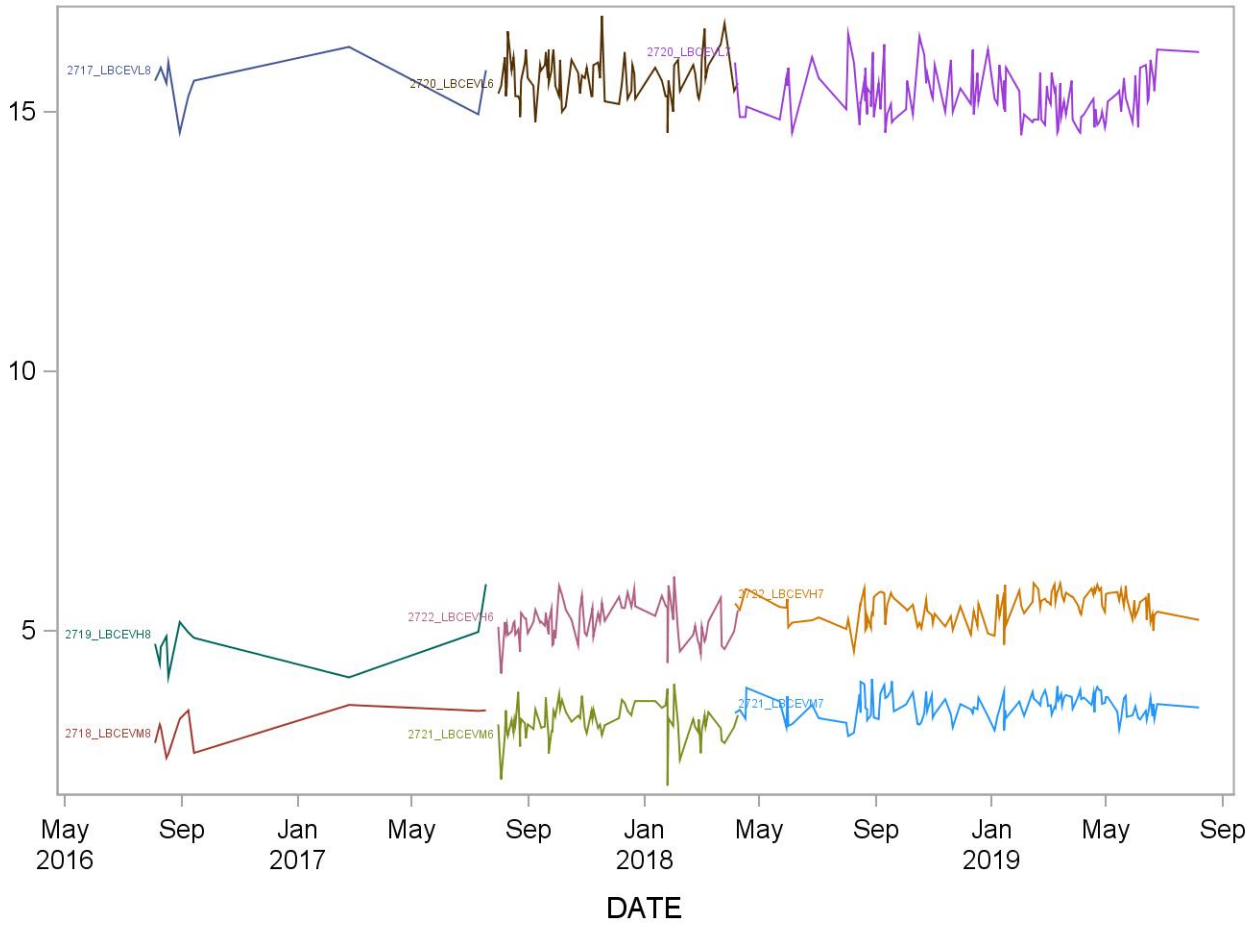
2015-2016 Summary Statistics and QC Chart
LBXVD3 (25OHD3 (nmol/L))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2719_LBC3VH8	9	04AUG16	18JUL17	88.444	1.231	1.4
2717_LBC3VL8	9	04AUG16	18JUL17	29.078	0.824	2.8
2718_LBC3VM8	9	04AUG16	18JUL17	50.883	1.384	2.7
2722_LBC3VH6	78	31JUL17	09APR18	87.915	1.901	2.2
2720_LBC3VL6	78	31JUL17	09APR18	29.476	0.563	1.9
2721_LBC3VM6	79	31JUL17	09APR18	51.081	0.996	1.9
2722_LBC3VH7	125	06APR18	07AUG19	93.492	1.783	1.9
2720_LBC3VL7	125	06APR18	07AUG19	30.527	0.681	2.2
2721_LBC3VM7	125	06APR18	07AUG19	53.934	1.007	1.9



2015-2016 Summary Statistics and QC Chart
LBXVE3 (epi-25OHD3 (nmol/L))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
2719_LBCEVH8	11	04AUG16	18JUL17	4.805	0.508	10.6
2717_LBCEVL8	11	04AUG16	18JUL17	15.568	0.468	3.0
2718_LBCEVM8	11	04AUG16	18JUL17	3.124	0.383	12.3
2722_LBCEVH6	79	31JUL17	09APR18	5.170	0.361	7.0
2720_LBCEVL6	80	31JUL17	09APR18	15.673	0.432	2.8
2721_LBCEVM6	80	31JUL17	09APR18	3.253	0.339	10.4
2722_LBCEVH7	124	06APR18	07AUG19	5.468	0.279	5.1
2720_LBCEVL7	124	06APR18	07AUG19	15.319	0.477	3.1
2721_LBCEVM7	124	06APR18	07AUG19	3.554	0.235	6.6



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Appendix A: Method Performance Documentation

Accuracy compared to Reference Material

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Reference material:	**NIST SRM 972a**
Analyte:	**25(OH)D3**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	**71.8**	68.2	70.0	69.3	70.4	69.4	69.9	1.02	1.46	-2.7
	2		69.7	70.7	71.8	68.8	70.2				
Level 2	1	**45.1**	46.6	44.3	43.8	45.8	43.5	44.3	1.49	3.36	-1.8
	2		44.1	42.4	45.6	41.9	44.9				
Level 3	1	**49.5**	48.1	50.0	49.4	49.5	48.1	48.5	0.96	1.97	-2.1
	2		48.5	47.1	47.3	48.8	47.9				
Level 4	1	**73.4**	67.8	69.5	68.4	71.3	70.9	70.0	1.54	2.21	-4.6
	2		68.1	70.1	70.3	71.9	72.0				

Accuracy compared to Reference Material

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Reference material:	**NIST SRM 972a**
Analyte:	**25(OH)D2**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	*1.30*	0.79	1.47	1.11	1.60	1.20	1.19	0.26	21.4	-8.3
	2		1.36	0.96	0.90	1.25	1.28				
Level 2	1	**2.00**	1.74	1.73	1.57	2.25	1.75	1.93	0.28	14.3	-3.6
	2		2.30	2.36	1.92	1.83	1.83				
Level 3	1	**32.3**	30.7	27.7	28.9	29.3	30.2	29.7	1.00	3.36	-8.0
	2		31.3	29.5	29.7	29.5	30.2				
Level 4	1	*1.30*	1.89	1.10	1.23	1.38	1.04	1.26	0.26	20.8	-2.9
	2		1.37	1.07	0.98	1.24	1.33				

Accuracy compared to Reference Material

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Reference material:	**CRM 001-004**
Analyte:	**25(OH)D2**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	3.01	3.50	3.24	2.56	3.01	3.20	3.07	0.28	9.0	2.1
	2		3.37	3.14	2.90	2.98	2.83				
Level 2	1	3.08	3.73	3.40	3.41	3.38	2.79	3.24	0.35	10.7	5.2
	2		3.64	3.37	2.81	2.86	3.02				
Level 3	1	13.16	13.3	13.1	13.4	13.4	11.5	12.9	0.62	4.8	-1.8
	2		13.1	12.7	13.6	12.7	12.4				
Level 4	1	16.74	17.4	16.2	17.8	18.1	17.5	17.4	0.96	5.5	3.6
	2		16.7	18.1	18.0	18.3	15.4				

Accuracy compared to Reference Material

Mean concentration should be within ±15% of the nominal value except at 3*LOD, where it should be within ± 20%

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Reference material:	**NIST SRM 972a**
Analyte:	**epi-25(OH)D3**

Reference material	Replicate	Nominal value	Measured concentration					Mean	SD	CV (%)	Difference from nominal value (%)
			Day 1	Day 2	Day 3	Day 4	Day 5				
Level 1	1	**4.50**	2.73	8.39	5.31	3.41	6.15	5.08	1.72	33.8	12.8
	2		3.83	6.36	4.70	3.80	6.10				
Level 2	1	**3.20**	1.61	4.27	3.72	2.55	4.14	3.27	0.90	27.6	2.3
	2		3.34	3.05	2.84	2.71	4.51				
Level 3	1	*2.90*	2.49	3.51	3.66	2.10	3.75	2.81	0.79	28.0	-3.1
	2		1.46	2.21	2.97	2.43	3.52				
Level 4	1	**64.8**	67.9	59.8	60.7	65.8	63.5	63.4	3.84	6.06	-2.2
	2		68.7	56.3	61.7	63.9	65.6				

Precision

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Analyte:	25(OH)D3

Quality material 1 (LS12473)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	31.10	31.10	31.10	0.0000	0.0000	1934.4200
2	28.70	28.30	28.50	0.0400	0.0400	1624.5000
3	28.90	29.30	29.10	0.0400	0.0400	1693.6200
4	29.60	30.40	30.00	0.1600	0.1600	1800.0000
5	29.20	27.30	28.25	0.9025	0.9025	1596.1250
6	29.90	31.10	30.50	0.3600	0.3600	1860.5000
7	30.20	28.70	29.45	0.5625	0.5625	1734.6050
8	29.40	30.00	29.70	0.0900	0.0900	1764.1800
9	29.70	28.70	29.20	0.2500	0.2500	1705.2800
10	28.50	27.50	28.00	0.2500	0.2500	1568.0000
Grand sum	587.6	Grand mean	29.38			

	Sum square	Mean Sq Erro	Std Dev	Rel Std Dev (%)
Within Run	5.3100	0.531	0.728697468	2.48
Between Run	17.542	1.949111111	0.842054366	2.87
Total	22.8520		1.113577817	3.79

Quality material 2 (HS12475)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	89.80	92.40	91.10	1.6900	1.6900	16598.4200
2	88.10	87.70	87.90	0.0400	0.0400	15452.8200
3	87.00	90.00	88.50	2.2500	2.2500	15664.5000
4	89.00	83.90	86.45	6.5025	6.5025	14947.2050
5	89.70	91.00	90.35	0.4225	0.4225	16326.2450
6	92.10	91.00	91.55	0.3025	0.3025	16762.8050
7	91.00	86.60	88.80	4.8400	4.8400	15770.8800
8	93.90	90.60	92.25	2.7225	2.7225	17020.1250
9	88.50	89.60	89.05	0.3025	0.3025	15859.8050
10	88.60	86.20	87.40	1.4400	1.4400	15277.5200
Grand sum	1786.7	Grand mean	89.335			

	Sum square	Mean Sq Erro	Std Dev	Rel Std
Within Run	41.025	4.1025	2.02546291	2.27
Between Run	65.4805	7.275611111	1.25958547	1.41
Total	106.5055		2.385174114	2.67

Precision

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Analyte:	25(OH)D2

Quality material 1 (LS12473)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	4.41	4.61	4.51	0.0100	0.0100	40.6802
2	4.80	3.83	4.32	0.2352	0.2352	37.2385
3	5.04	4.91	4.98	0.0042	0.0042	49.5013
4	4.40	4.34	4.37	0.0009	0.0009	38.1938
5	4.36	4.86	4.61	0.0625	0.0625	42.5042
6	4.95	4.98	4.97	0.0002	0.0002	49.3025
7	4.66	5.04	4.85	0.0361	0.0361	47.0450
8	5.11	5.47	5.29	0.0324	0.0324	55.9682
9	4.50	4.38	4.44	0.0036	0.0036	39.4272
10	4.79	4.22	4.51	0.0812	0.0812	40.5901
Grand sum	93.66	**Grand mean**	4.683			

	Sum square	Mean Sq Erro	Std Dev	Rel Std Dev (%)
Within Run	0.9328	0.09328	0.305417747	6.52
Between Run	1.84102	0.204557778	0.235878971	5.04
Total	2.77382		0.385900102	**8.24**

Quality material 2 (HS12475)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	20.90	19.90	20.40	0.2500	0.2500	832.3200
2	19.40	20.10	19.75	0.1225	0.1225	780.1250
3	18.80	18.90	18.85	0.0025	0.0025	710.6450
4	20.10	20.30	20.20	0.0100	0.0100	816.0800
5	18.60	19.40	19.00	0.1600	0.1600	722.0000
6	20.70	18.80	19.75	0.9025	0.9025	780.1250
7	19.40	18.40	18.90	0.2500	0.2500	714.4200
8	19.90	21.10	20.50	0.3600	0.3600	840.5000
9	20.00	20.30	20.15	0.0225	0.0225	812.0450
10	20.50	19.10	19.80	0.4900	0.4900	784.0800
Grand sum	394.6	**Grand mean**	19.73			

	Sum square	Mean Sq Erro	Std Dev	Rel Std
Within Run	5.14	0.514	0.716937933	3.63
Between Run	6.882	0.764666667	0.354024481	1.79
Total	12.022		0.799583225	**4.05**

Precision

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Analyte:	epi-25(OH)D3

Quality material 1 (LS12473)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	18.10	17.90	18.00	0.0100	0.0100	648.0000
2	16.90	17.10	17.00	0.0100	0.0100	578.0000
3	17.30	15.50	16.40	0.8100	0.8100	537.9200
4	15.10	15.90	15.50	0.1600	0.1600	480.5000
5	14.70	15.10	14.90	0.0400	0.0400	444.0200
6	15.90	16.60	16.25	0.1225	0.1225	528.1250
7	16.10	14.90	15.50	0.3600	0.3600	480.5000
8	16.00	14.60	15.30	0.4900	0.4900	468.1800
9	14.90	14.90	14.90	0.0000	0.0000	444.0200
10	15.10	14.80	14.95	0.0225	0.0225	447.0050
Grand sum	317.4	Grand mean	15.87			

	Sum square	Mean Sq Erro	Std Dev	Rel Std Dev (%)
Within Run	4.05	0.405	0.636396103	4.01
Between Run	19.132	2.125777778	0.927571501	5.84
Total	23.182		1.124895057	**7.09**

Quality material 2 (HS12475)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	5.26	5.11	5.19	0.0056	0.0056	53.7685
2	5.02	4.50	4.76	0.0676	0.0676	45.3152
3	5.42	5.02	5.22	0.0400	0.0400	54.4968
4	6.17	5.55	5.86	0.0961	0.0961	68.6792
5	6.75	5.47	6.11	0.4096	0.4096	74.6642
6	4.96	5.42	5.19	0.0529	0.0529	53.8722
7	5.70	5.06	5.38	0.1024	0.1024	57.8888
8	4.79	4.12	4.46	0.1122	0.1122	39.6941
9	5.79	5.31	5.55	0.0576	0.0576	61.6050
10	5.28	4.69	4.99	0.0870	0.0870	49.7005
Grand sum	105.39	Grand mean	5.2695			

	Sum square	Mean Sq Erro	Std Dev	Rel Std
Within Run	2.06215	0.206215	0.454109018	8.62
Between Run	4.331745	0.481305	0.370870597	7.04
Total	6.393895		0.586310498	**11.13**

Stability

The initial measurement can be from the same day for all stability experiments.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: example: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: example: original samples (not yet prepared for instrument analysis) stored at room temperature for 1 day
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: example: processed samples (ready for instrument analysis) stored at room temperature for 1 day
Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Analyte:	**25(OH)D3**

Quality material 1	LS12473		LS12473		LS10473		LS12473	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	29.5	28.3	29.5	29.6	30.9	29.2	29.9	29.5
Replicate 2	28.3	28.7	28.3	31.3	29.1	29.5	29.7	28.3
Replicate 3	29.9	27.6	29.9	30.6	33.9	30.6	29.6	29.9
Mean	29.2	28.2	29.2	30.5	31.3	29.8	29.8	29.2
% difference from initial measurement	--	**-3.53**	--	**4.33**	--	**-4.90**	--	**-1.77**

Quality material 2	HS12475		HS12475		HS10475		HS12475	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	86.6	87.0	86.6	92.0	86.4	83.0	96.6	86.6
Replicate 2	83.8	86.3	83.8	86.9	84.6	87.9	92.9	83.8
Replicate 3	87.9	87.7	87.9	87.5	86.7	90.2	96.9	87.9
Mean	86.1	87.0	86.1	88.8	85.9	87.0	95.5	86.1
% difference from initial measurement	--	**1.05**	--	**3.14**	--	**1.33**	--	**-9.80**

Stability

The initial measurement can be from the same day for all stability experiments.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: example: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: example: original samples (not yet prepared for instrument analysis) stored at room temperature for 1 day
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: example: processed samples (ready for instrument analysis) stored at room temperature for 1 day
Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Analyte:	**25(OH)D2**

Quality material 1	LS12473		LS12473		LS10473		LS12473	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	5.04	4.77	5.04	4.75	73.4	69.5	5.53	5.04
Replicate 2	4.79	4.61	4.79	5.04	70.6	67.3	4.31	4.79
Replicate 3	4.15	4.28	4.15	4.92	70.3	65.1	5.76	4.15
Mean	4.66	4.55	4.66	4.90	71.5	67.3	5.20	4.66
% difference from initial measurement	--	**-2.29**	--	**5.22**	--	**-5.82**	--	**-10.4**

Quality material 2	HS12475		HS12475		HS10475		HS12475	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	19.0	19.1	19.0	20.0	45.4	41.4	20.5	19.0
Replicate 2	18.3	19.9	18.3	18.9	45.8	39.3	22.4	18.3
Replicate 3	17.8	19.1	17.8	18.9	43.6	40.9	22.5	17.8
Mean	18.4	19.4	18.4	19.3	44.9	40.5	21.8	18.4
% difference from initial measurement	--	**5.44**	--	**4.90**	--	**-9.77**	--	**-15.7**

Note: HS12475 in long-term stability for 25(OH)D2 was slightly outside of ±15% of nominal concentration. Since the calibration matrices used were different for the initial (solvent-based) and final (serum-based) measurements, we will re-collected this data in two years using serum-based calibrators.

Stability

The initial measurement can be from the same day for all stability experiments.

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: example: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: example: original samples (not yet prepared for instrument analysis) stored at room temperature for 1 day
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: example: processed samples (ready for instrument analysis) stored at room temperature for 1 day
Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L
Analyte:	**epi-25(OH)D3**

Quality material 1	LS12473		LS12473		LS10473		LS12473	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	16.3	15.8	16.3	15.1	44.0	45.5	15.3	16.3
Replicate 2	14.7	15.0	14.7	16.2	47.1	41.8	16.7	14.7
Replicate 3	16.8	14.2	16.8	15.2	47.6	45.5	15.1	16.8
Mean	15.9	15.0	15.9	15.5	46.2	44.3	15.7	15.9
% difference from initial measurement	--	**-5.86**	--	**-2.72**	--	**-4.26**	--	**1.44**

Quality material 2	HS12475		HS12475		HS10475		HS12475	
	Initial measurement	Three freeze-thaw cycles	Initial measurement	Bench-top stability	Initial measurement	Processed sample stability	Initial measurement	Long-term stability
Replicate 1	5.55	5.04	5.55	4.65	19.6	19.1	4.72	5.55
Replicate 2	4.40	5.02	4.40	5.10	19.4	19.5	5.55	4.40
Replicate 3	4.89	5.03	4.89	5.00	22.5	18.4	6.08	4.89
Mean	4.95	5.03	4.95	4.92	20.5	19.0	5.45	4.95
% difference from initial measurement	--	**1.68**	--	**-0.61**	--	**-7.46**	--	**-9.21**

LOD, specificity and fit for intended use

Method name:	25-hydroxyvitamin D LC-MS/MS
Method #:	4027
Matrix:	Serum
Units:	nmol/L

Analytes	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
25(OH)D2	2.05	yes	yes
25(OH)D3	2.23	yes	yes
C3-epi-25(OH)D3	1.64	yes	yes

Appendix B: Ruggedness Testing

A. Sample Preparation Conditions – Manual vs. Hamilton vs. Hybrid

(1) 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.

(2) Proposal: Process samples using three different methods

a) Manually using all manual pipettes for liquid handling, dry ice bath freezing, manual pour-off of all hexane, manual reconstitution, and manual syringe filtration.

b) 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.

c) 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 (one run per factor).

(3) Conclusion: Alterations to the pipetting technique (manual, robotic, or hybrid method) may be done without adverse effect. In general, when concentrations are ≥ 10nmol/L, either of these methods may be used.

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 VID2:* 3.16, 5.84, **63.2**, 7.54 *SRM972 VID3:* **66.1**, **34.7, 46.4, 77.6** *SRM972 VID3E:* 4.99, 4.12, 2.50, **95.6**	VID2 mean=2.0 VID2: 2.2, 1.4, **1.0**, 1.3 VID3 mean=1.0 VID3: **1.1, 1.3, 1.0, 0.9** VID3E mean=1.4 VID3E: 1.4, 2.1, 0.9, **1.0**
Hamilton preparation only	All Robotic pipetting No syringe filters	*SRM972 VID2:* 1.95, 4.62, **70.0**, 5.59 *SRM972 VID3:* **64.6**, **29.2, 45.7, 80.1** *SRM972 VID3E:* 3.74, 0.75, 0.75, **83.9**	VID2 mean=0.94 VID2: 0.9, 0.9, **1.1**, 0.9 VID3 mean=0.94 VID3:**1.0, 0.9, 0.9, 0.9** VID3E mean= 1.0 VID3E: 1.0, 1.0, 1.1, **1.0**
Hybrid preparation Manual+Hamilton	Robotic pipetting 100 µL manual pipette 0.45 µm syringe filter	*SRM972 VID2:* 0.97, 3.65, **68.3**, 5.84 *SRM972 VID3:* **49.4, 28.1, 43.7, 69.6** *SRM972 VID3E:* 3.24, 1.50, 1.75, **93.4**	VID2 mean=0.9 VID2: 0.7, 0.9, **1.1**, 1.0 VID3 mean=0.9 VID3: **0.8, 0.9, 0.9, 0.8** VID3E mean=0.8 VID3E: 0.9, 0.8, 0.6, **1.0**

B. Sample Preparation Conditions – Plate Dryer vs. SpeedVac

(1) 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.

(2) 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)

(3) 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 25OHD2. 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.

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 qualifier 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 quantifier to the qualifier ion within ± 30% of the average peak area ratio of the quantifier to the qualifier 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	1.89 ± 30%	2.77 ± 30%	2.82 ± 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

In March 2014, we recalculated ion ratios as the qualifier divided by the quantifier to synchronize with the MS/MS community.

In December 2017, confirmation ion ratio limits (Rule 1) were re-calculated using NHANES 2013-2014 results ≥LOQ to reflect LC-MS/MS instrument-specific ratios. The CDC laboratory currently uses two LC-MS/MS systems: Aten and Helios.

Analyte	LOD (nM)	LOQ (nM)	LC-MS/MS	NHANES 2013-2014*				Confirmation Ion Ratios for results ≥ LOQ**		
				Total No. Results	No. Results ≥LOQ	No. Results ≥LOQ & used in Calculation	% Results ≥LOQ & used in Calculation	Mean	30% LL	30% UL
VID2	2.05	6.15	Aten	6644	503	500	8%	0.59	0.41	0.77
			Helios	4368	279	279	6%	0.55	0.39	0.72
VID3	2.23	6.69	Aten	6644	6590	6589	99%	0.48	0.33	0.62
			Helios	4368	4353	4353	100%	0.30	0.21	0.39
VID3E	1.64	4.92	Aten	6676	1547	1544	23%	0.47	0.33	0.61
			Helios	4368	1042	1042	24%	0.29	0.21	0.38

*NH13-14 Blind QCs are excluded. All pass/fail runs and all comment codes are included, but extreme outlier points are excluded.

**Peaks area ratios is the peak area of qualifier ion divided by the peak area of quantifier ion.

References for Appendix C:

1. Kushnir, MS, Rockwood, AL. High-sensitivity tandem mass spectrometry assay for serum estrone and estradiol. Am J Clin Pathol. 2008; 129:530-539.

2. FAO/WHO Codex Committee on Pesticide Residue, Codex Alimentarius CAC/GL 56-2005.