Laboratory Procedure Manual

Analyte: **25-Hydroxyvitamin D3, C3-epimer-25-Hydroxyvitamin D3, and 25-Hydroxyvitamin D_2**

Matrix: **Serum**

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

Method No: 4027.10

Revised: December 2022

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

contact: Ekaterina Mineva, Ph.D.
Phone: 404-498-1856
Email: jpu8@cdc.gov

James L. Pirkle, M.D., Ph.D.
CLIA Laboratory Director, Centers for Disease Control and Prevention

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.

Images are included in this document as visual aids for certain topics. They are intended to be representative images only and should not be construed as absolute references. Discrepancies between the images in this document and the actual application design are not a cause for revisions to this document.

Public Release Data Set Information

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

Lab Number	Analyte	SAS Label (and SI units)
VID_L	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 D_3 (nmol/L)

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 utilizes high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) after liquid-liquid extraction 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.

Serum samples are first treated by the addition of a 67% methanol solution containing three internal standards (IS) and additional solution of approximately 72% methanol in water to allow enough volume to partition the organic and aqueous phases during liquid-liquid extraction; this is followed by the addition of hexane. Analytes are extracted from the aqueous phase into the hexane layer, which is then dried under vacuum. The extract is re-dissolved with approximately 72% methanol in water. An aliquot of the extract is injected onto the pentafluorophenyl (PFP) column and gradient elution is used for the separation of 25OHD$_3$, epi-25OHD$_3$, 25OHD$_2$, and the IS, hexadeuterium-25-hydroxyvitamin D$_3$, trideuterium-3-epi-25-hydroxyvitamin D$_3$, and trideuterium-25-hydroxyvitamin D$_2$ during a 13-minute run. The initial mobile phase composition is approximately 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 initial mobile phase. Detection is performed by using a triple quadrupole tandem mass spectrometer (Thermo TSQ Altis 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 or comparable 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. 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.

Additional information on hazard identification, risk evaluation, and risk mitigation for this method can be found in the method risk assessment form.

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 (Indigo Ascent, Carmel, IN). 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 STARLIMS (Hollywood, Florida) customized database for review of the data, statistical evaluation of QC/QA data, and approval of the results. See "**JA-4027-DR-01-Computerization & Data System Management" in Appendix B, section D** for the description of data transfer, review, and approval.

(C) The instrument raw and results files (including all patient, QC, and calibration data) on instrument computer hard drives are backed up daily to the CDC network automatically via an ISLE network. This is verified weekly by 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™. Specimens collected in the field should be kept refrigerated (+2°C to +8°C) and protected from light. After processing, specimens should be frozen and shipped on dry ice by overnight mail. Once received, serum specimens should be stored frozen during 'in-processing', which is typically completed within less than 4 hours and then stored frozen at ≤-50°C for up to 15 business days until they are transferred to the testing laboratory. For longer storage, specimens should be stored deep frozen (-50°C to -90°C). However, 25OHD is very stable and serum samples can also be kept frozen (-10°C to -50°C) for at least a year prior to analysis. Several freeze-thaw cycles do not seem to adversely affect the assay, although more than three freeze-thaw cycles should be avoided.

(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) Specimens should generally arrive frozen. Refrigerated samples (+2°C to +8°C) may be used, provided they are brought promptly from the site of collection.

(F) Moderately hemolyzed specimens may be used because red blood cells do not interfere (+ or -) with 25OHD results.

(G) Specimen handling conditions are outlined in the Policies and Procedures Manual of the Division of Laboratory Sciences (copies are available in the Nutritional Biomarkers Laboratory and an electronic copy of this file is located on a local shared drive). The protocol discusses collection and transport of specimens and the special equipment required. 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.

(H) The criteria for unacceptable specimens are insufficient sample volume for at least one analysis (<150 µL for manual pipetting), suspected contamination such as leaking, or damaged sample container. These samples are assigned an appropriate comment code and/or description and are set "no reportable" (code 98). 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.

(I) A series of standard comment codes are available in the LIMS database to identify any issues related to sample quality. These codes can be used, along with text descriptions, to document why a result was not reported (specimen rejection) or that a result should be interpreted with caution based on the sample quality.

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

Chemicals and other materials used in the preparation of reagents, calibrators, and quality control materials are tracked as indicated in Job Aid "**JA-4027-R&S-01-Reagents & Standards Tracking**" **in Appendix B, section B**. To facilitate tracking of solvents and other chemicals transferred from the original manufacturer containment into a secondary container (e.g., solvent bottle), include the expiration date provided by the manufacturer or the lot number on the secondary container.

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 or smaller quantities if desired.

Any water used to prepare reagents refers to deionized water with a resistivity of at least 18 MΩ·cm. Reagent-grade ethanol, HPLC-grade hexane, and HPLC-grade or LC-MS-grade 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 – 72% Methanol (if pre-mixing solvents)

   Obtain 2 clean dry graduated cylinders. To one cylinder add 280 mL water and to the other cylinder add 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 ambient temperature (+15°C to +30°C) for 3 months when kept tightly capped.

2) HPLC Mobile Phase – 100% Methanol

   Methanol is added to a clean 1000-mL bottle. This solution is stable at ambient temperature (+15°C to +30°C), expiration date provided by manufacturer.

3) HPLC Mobile Phase – 100% Water (if pump-mixing solvents)

   DI-Water is added to a clean 1000-mL bottle. This solution is stable at ambient temperature (+15°C to +30°C) for 1 week when kept tightly closed in an amber bottle.

4) HPLC Needle Wash – 72% Methanol

   Match needle wash composition to that of the initial mobile phase. Methanol (720 mL) is added to a 1000-mL bottle. Water (280 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 ambient temperature (+15°C to +30°C) for 3 months when kept tightly capped.

5) HPLC Seal Wash – 10% Methanol

   Water (900 mL) is added to a 1000-mL bottle. Methanol (100 mL) is added to the same bottle. This solution is stable at ambient temperature (+15°C to +30°C) for 1 month when kept tightly capped.

6) 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 for 2 weeks when kept refrigerated (+2°C to +8°C). It is used for the matrix blank (4% albumin in 0.01M PBS).

7) 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 refrigerated (+2°C to +8°C) or prepare a large batch as needed, aliquot 500 µL into 1.8-mL cryovials and store deep frozen (-50°C to -90°C).

8) 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 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 refrigerated (+2°C to +8°C) and is prepared as needed.

B. Standards Preparation

The stock solutions and calibration solutions are stored in 1.8-mL polypropylene cryovials deep frozen (-50°C to -90°C) and in our experience are stable for many years.

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. This solution is stable for at least 5 years when kept deep frozen (-50°C to -90°C) and tightly capped.

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. This solution is stable for at least 5 years when kept deep frozen (-50°C to -90°C) and tightly capped.

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. This solution is stable for at least 5 years when kept deep frozen (-50°C to -90°C) and tightly capped.

For details on how to confirm the concentration of each stock solution by UV absorption refer to **Appendix C** 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 deep frozen (50°C to -90°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 reduces 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)		
	$25OHD_2$ stock	$25OHD_3$ stock	epi-$25OHD_3$ stock	Intermediate Stock	Matrix	$25OHD_2$	$25OHD_3$	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)		
	25OHD$_2$ stock	25OHD$_3$ stock	epi-25OHD$_3$ stock	calibrator	Matrix (100% ethanol)	matrix (serum)	matrix (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).

The serum-based calibrators are stable for at least 6 years when kept deep frozen (-50°C to -90°C) and tightly capped.

2) Internal Standard Solutions (Stock and Working)

The internal standards arrive from the vendors in powder form at ambient temperature (+15°C to +30°C).

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. This solution is stable for at least 10 years when kept deep frozen (-50°C to -90°C) and tightly capped.

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. This solution is stable for at least 10 years when kept deep frozen (-50°C to -90°C) and tightly capped.

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. This solution is stable for at least 10 years when kept deep frozen (-50°C to -90°C) and tightly capped.

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. This solution is stable for at least 5 years when kept deep frozen (-50°C to -90°C) and tightly capped.

C. Preparation of Quality Control Materials

Low, medium, and high bench quality control 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 4-20 nmol/L for 25OHD$_2$, 30-95 nmol/L for 25OHD$_3$ and 3-15 nmol/L for 3-epi-25OHD$_3$.

To prepare pools, first prescreen the 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 deep frozen (-50°C to -90°C). Deep frozen aliquots have no known expiration date. Note, sometimes it is necessary to spike serum with analytes to achieve the desired concentrations. For more detailed information on the preparation of QC materials, homogeneity testing, and characterization refer to **SOP "NBB-OC-LABOP.01.01 QC Materials"**.

At least 6 levels of blind QC pools may be prepared in the same way that bench QC pools are prepared. Store the pools deep frozen (-50°C to -90°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.

(1) Ascentis Express F5 2.1 x 150 mm: 2.7 µm particle size column (Sigma-Aldrich, St. Louis, MO)
(2) KrudKatcher Ultra Inline HPLC filter, 0.5 µm depth x 0.004 in ID (Phenomenex, Torrance CA)
(3) 2.1 mm ID x 2 µm inline filter (Thermo Scientific, Belefonte, PA)
(4) 13 x 100 mm Disposable glass culture tubes (Corning Glassworks, Corning, NY)
(5) 5¾" Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)
(6) Solvent filters, 0.45 µm pore size (Millipore Corp, Medford, MA)
(7) N-Dex nitrile examination gloves (Best Manufacturing Corp, Menlo, GA)
(8) 0.45 µm Syringe tip PVDF hydrophilic filter (4 mm diameter) (obtained from various sources)
(9) Plastic tuberculin syringes (obtained from various sources)
(10) 1.8-mL Polypropylene cryovials (Nalgene Company, Rochester, NY)
(11) Various glass beakers, volumetric flasks, graduated cylinders and bottles (class A glassware)

(12) Methanol, HPLC grade (Tedia, Fairfield, OH or Honeywell, Morris Plains, NJ)
(13) Methanol, LC-MS grade (VWR, Radnor, PA)
(14) Hexane, HPLC grade (Fisher Scientific, Pittsburg, PA)
(15) Ethanol, HPLC grade (obtained from various sources)
(16) Albumin from bovine serum (Sigma, St. Louis, MO)
(17) Extended Mass Range (EMRS) Calibration Solution (Fisher Scientific, Pittsburg, PA)
(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* if a product substitution is made. Equivalent performance must also be demonstrated in accordance with *DLS Policies and Procedures* when multiple analysis systems are used in parallel, even if they are of the exact same type. To provide adequate throughput for this method, as well as backup instrumentation during times of repair and maintenance, we may utilize multiple LC-MS/MS systems of the Thermo Altis type. The comparisons involve analyzing several samples on each of the instruments and assessing the resulting Pearson correlation coefficients. For details, see Job Aid "**JA-4027-I-01-LC-MS/MS Instrument Comparison and System Verification**" **in Appendix B, section C**.

(1) Thermo TSQ-Altis mass spectrometer, with Xcalibur software (ThermoElectron Corp, West Palm Beach, FL)
(2) Thermo Vanquish 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) UV-Vis 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) Plate dryer (Biotage, Charlotte, NC)
(17) Hamilton Microlab STARlet (Hamilton, Reno, NV)

7. Calibration and Calibration Verification Procedures

A. 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 1 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 their respective 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, 6- to 8-point linear curves (with a set of calibrators each at the beginning and end of the run), not forced through zero, are generated. The concentrations (x-axis) are calculated from the regression equation based on the response ratios (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 are used as described above.

(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 ambient temperature changes 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 through the full extraction process as described in section 8a and 8b. Peak area ratios are used as described above.

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.

The above conditions are met for the current method and therefore no additional calibration verification is required. However, calibration verification using international reference materials is conducted at least every 6 months to monitor the performance of the method. Refer to "**JA-4027-G-01-Calibration and Calibration Verification" in Appendix B, section A**.

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. We also use 25OHD reference materials value-assigned by the Ghent University reference laboratory, Ghent, Belgium.

The CDC laboratory participates in multiple proficiency testing programs for 25OHD. The primary one is sponsored by Vitamin D External Quality Assessment Scheme (DEQAS, London, UK); others are sponsored by National Institutes of Standards and Technology (NIST, Gaithersburg, MD), CDC's Vitamin D Standardization and Certification Program (VDSCP, Atlanta, GA), and College of American Pathologists (CAP, Northfield, IL). Other proficiency testing programs beyond DEQAS are optional and may not be carried out regularly.

B. Instrument Calibration and Calibration Verification

1) Thermo LC-MS/MS

The calibration of the instrument is generally scheduled on a semi-annual basis, as a part of the preventive maintenance, and is performed by the service engineer from ThermoElectron Corp. If necessary, user recalibrates using the calibration standards and by following the instructions contained in the Operator's Manual (see STARLIMS) or submit a service ticket. See "**JA-4027-I-02-Thermo LC-MS/MS System Operation and Maintenance" in Appendix B, section C** for detailed instructions.

Compound-dependent optimization of the instrument (TSQ Altis) is generally done initially when setting up the method on a new instrument. Users may periodically conduct optimizations when major service is conducted. See "**JA-4027-I-02-Thermo LC-MS/MS System Operation and Maintenance" in Appendix B, section C** 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 the Volume Field Verification kit from Hamilton. Users can also run the optional daily and weekly performance checks (performed as needed).

Refer to "**JA-4027-I-06-Hamilton Microlab STARlet Liquid Handler Calibration Verification using the Volume Field Verification (VFV) Kit" in Appendix B, section C** for detailed instructions on the liquid handler calibration verification procedure using the Volume Field Verification (VFV) kit.

3) Pipettes

Pipette calibration verification (e.g., for air displacement and positive displacement pipettes) is performed biannually. Typically, one calibration verification is done by a certified company and one by the analyst gravimetrically using a calibrated analytical balance.

4) Varian UV-Vis spectrophotometer

Performance testing is done three times per year by participation in the CAP instrumentation survey. Additionally, every time the instrument is turned on there are internal diagnostics that are run. Calibration verification using certified filters is performed at least annually, generally twice per year. Calibration verification of the certified filters is performed externally every other year.

5) Balances

On site calibration is performed annually by a certified company. Calibration verification is performed by the analyst as needed using certified weights.

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 deep frozen storage (-50°C to -90°C). Allow vials to reach ambient temperature (+15°C to +30°C), then gently vortex them prior to pipetting. If using a multi-tube vortexer, vortex at 2,300 setting for 1 minute. 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 pre-programmed for the procedure. For programming instructions see Hamilton Microlab STARlet Operators Manual (see STARLIMS) and see "**JA-4027-I-03-Hamilton Microlab STARlet Liquid Handler Operation for liquid-liquid extraction**" **in Appendix B, section C** 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 72% 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 ambient temperature (+15°C to +30°C). 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 ambient temperature (+15°C to +30°C).

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

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

(6) Cover the plate with a pre-slit silicone plate cover and load into the Vanquish autosampler chamber set to 7°C.

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

B. Sample Preparation (manual preparation)

(1) Prepare an ethanol/dry ice bath (temperature is between -20°C and -50°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 manufacturer's instruction manual (see STARLIMS) for specific steps for drying hexane.

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

(7) Take the reconstituted 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 pre-slit silicone plate cover and load into the Vanquish autosampler chamber set at 7°C.

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

C. LC-MS/MS Analysis

(1) HPLC columns are generally held at 30°C under gradient conditions. A variety of columns may be used for this assay and are not limited to the one 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 the following analytical column:

Column	Mobile Phase Composition	Flow Rate
Ascentis Express F5	~72% methanol : 28% water (A) with introduction of 100% methanol (B) until it reaches 80% A to 20% B (Curve: 5)	~350 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$_2$ (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 Altis 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* 383.25→365.25; **epi-25OHD$_3$**, *m/z* 383.25→365.25; **25OHD$_2$**, *m/z* 395.3→377.3; Internal Standards: ***d6*-25OHD$_3$** *m/z* 389.25→371.25, ***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 generally in <9 minutes.

Refer to **Appendix D** for MS Transitions and LOD by Analyte in STARLIMS.

(4) Qualitative (Confirmation) Ions: Alternative product ions are measured to confirm peak identity. The ratio of the area of the qualitative ion ÷ quantitative ion is monitored. The following qualitative transitions are recorded: $25OHD_3$, m/z 383.25→105; epi-$25OHD_3$, m/z 383.25→105; $25OHD_2$, 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 E**.

(5) The MS instrument settings are generally as follows: Ion Source Type = APCI, Polarity = Positive, Spray Current = Static, Positive Ion Discharge Current = 1 µA, Negative Ion Discharge Current = 10 µA, Sheath Gas (Arb) = 25, Auxiliary Gas (Arb) = 2, Sweep Gas (Arb) = 0.4, Ion Transfer Tube Temperature = 325°C, Vaporizer Temperature = 350°C, Source Fragmentation (analyte-dependent) = 27-29, Collision Energy (analyte-dependent) = 13-43, Dwell Time = 100 ms, and CID Gas = 1.5 mTorr.

(6) A portion (50 µL) of the extract is injected. Generally, the first 5 min of each injection is diverted to waste, data are collected from 5-8.5 min, and the effluent is again directed to waste for the remainder of the run. There is a 1-2 min wash with 100% methanol directly following data collection. Each injection takes approximately 13 mins to finish.

D. Instrument Preparation

(1) HPLC Preparation

a) Refer to "**JA-4027-I-02-Thermo LC-MS/MS System Operation and Maintenance**" **in Appendix B, section C** for detailed instructions on how to set up the instrument, check its performance, and start a run.

b) Mobile phase solvents: Line #1: 72% methanol in water, and Line #2: 100% methanol, Line #3: 100% water

c) Needle wash solution: 72% methanol in water

d) Seal wash solution: 10% methanol in water

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

(2) Mass Spectrometer Preparation

Refer to **"JA-4027-I-02-Thermo LC-MS/MS System Operation and Maintenance" in Appendix B, section C** 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 "**JA-4027-I-02-Thermo LC-MS/MS System Operation and Maintenance" in Appendix B, section C** 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. Processing and reporting a run

(1) Refer to "**JA-4027-DR-01-Computerization & Data System Management" in Appendix B, section D** for general information on peak integration and chromatographic review. Instead of using the Thermo Xcalibur software to process a run, the Indigo Ascent Automated Integration Software can be utilized to perform integrations and quantitation (current practice). For specific information on how to use Ascent, refer to "**JA-4027-DR-02 Ascent Data Review" in Appendix B, section D**.

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

G. Data Review and Calculations

Refer to "**JA-4027-DR-01-Computerization & Data System Management" in Appendix B, section D** for information on how to conduct multi-level data review using STARLIMS. For more detailed information on data review and associated criteria refer to "**JA-4027-DR-03-STARLIMS Data Review and Criteria" in Appendix B, section D**. For a graphical presentation of the data review process, refer to "**JA-4027-DR-04-STARLIMS Data Review Flowchart" in Appendix B, section D**.

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, which is the case for about 80% of NHANES samples. The imputed value is the LOD divided by the square root of 2.

Report an imputed value for C3-epi-$25OHD_3$ when it is <LOD.

Check calibration curves for each analyte. Coefficients of determination should be R^2 >0.98.

Review each chromatogram and do manual peak selection and integration when necessary. Code this action in STARLIMS.

Check bench QC results for each analyte against bench 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.

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

H. System Maintenance

1) Thermo TSQ Altis

Preventative maintenance is performed annually or semi-annually by an authorized service engineer. Analyst performs maintenance as described in "**JA-4027-I-02-Thermo LC-MS/MS System Operation and Maintenance" in Appendix B, section C** as needed due to decreasing sensitivity or signal loss. Analyst may also setup a schedule for certain operations such as cleaning various components, ballast pumps, etc. Analyst may check mass calibration as needed.

Refer to "**JA-4027-I-05-Thermo LC-MS/MS Checking Mass Calibration on TSQ-Altis**" and "**JA-4027-I-04-Thermo LC-MS/MS Shutdown Instructions" in Appendix B, section C** for detailed instructions.

2) Vanquish UHPLC system

Preventative maintenance is performed annually or semi-annually by an authorized service engineer. Analyst performs maintenance as described in "**JA-4027-I-02-Thermo LC-MS/MS System Operation and Maintenance**" **in Appendix B, section C** as needed.

3) Hamilton Microlab Starlet

Twice a year, a Hamilton Service Engineer performs preventative maintenance.

4) Varian UV-Vis spectrophotometer

Preventative maintenance and calibration of the instrument are performed annually by an authorized service engineer. Calibration verification is performed annually using internal diagnostics and a set of certified filters.

I. CDC Modifications

This method was published in 2011 **[8]**. This document represents the tenth 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 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 $25OHD_2$ 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. This method also permits the use of cal8 for repeat analysis on specimens that exceed cal7 without dilution. 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 with *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:

$25OHD_3$	0.439 - ~300 nmol/L
3-epi-$25OHD_3$	0.755 - ~80 nmol/L
$25OHD_2$	2.216 - ~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.

In 2012, an open label blind QC program was instituted. Open label blind QC specimens means that 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 one can choose from at least 6 different pools, and the analyte concentrations are similar to those found in patient samples. The frequency of blind QC specimens in a run is typically 1 in every 20 patient specimens analyzed.

After a run is completed, used blind QC are removed from the run, marked with a black dot on the cap to indicate that the vial has been thawed, and returned to the blind QC box. This helps to identify which vials have been used. If a run needs to be repeated, the same blind QC can be inserted as in the initial run.

The use of blind QCs is optional but encouraged. Blind QCs are used in this method as a supplementary tool to assist in monitoring accuracy, precision, and aid in detecting errors such as sample misalignment; they are not used as part of the primary control procedures to determine if a run is out of control.

Bench Quality Controls

Bench QC specimens are prepared from a minimum of two pools that represent low and high levels of serum 25OHD. This assay typically uses 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 using a multi-rule quality control system [**10**] based on their characterization data, namely: the pool mean; the pooled within-run standard deviation associated with individual QC results measured in the same run (S_w); the standard deviation associated with individual QC results (S_i); and the standard deviation associated with run mean QC results (S_m). QC rules have been designed to accommodate the use of 1–3 different QC pools during a run, the use of 1–2 measurements of each pool per run, and as many instruments as needed. These QC rules are described in the DLS Policies and Procedures Manual and a relevant selection applicable to this assay is shown below. The system is declared "in control" if all individual QC results are within 2S limits; the run is accepted. If not, then the rules shown below are applied and the run is rejected if any condition is met; the run is 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 2 S_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 2 S_m limit, reject run if:

1. 1 3S Rule—Run mean is outside a 3 S_m limit or
2. 2 2S Rule—Two or more of the three run means are outside the same 2 S_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 2 S_i limit, reject run if:

1. Outlier—One individual result is beyond the characterization mean ± 4 S_i or
2. 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 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. The SAS QC program is used to monitor the QC performance over time for potential shifts, trending, or changes in assay precision. For assays performed routinely, quarterly statistics (mean, SD, CV) are calculated for each pool and compared to the characterization target values. For assays performed infrequently, statistics are calculated at least annually. As more QC data become available (covering multiple lots of reagents, multiple analysts, etc.), the initial QC limits can be reevaluated and updated. QC limits can also be reevaluated and updated as a result of a non-conforming event when the assay shows a higher than expected out of control rate and the root cause investigation does not reveal a correctable course of action to bring the assay back into control. This needs to be documented by a CAPA in STARLIMS.

While a study is in progress, QC results are stored in the 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 and submitted as a study QC report in STARLIMS for review by the laboratory chief, branch chief, and a DLS statistician.

B. Sample QC Criteria

Sample QC is a set of criteria used to evaluate the quality of individual test results within a run, and to evaluate the quality of the calibration curves associated with a run. In addition to the sample QC criteria set forth in the DLS Policies and Procedures Manual that pertain to the reportable range of concentration results and calibration curves, sample QC criteria are also established for method-specific concentration and non-concentration data associated with an individual test result.

The method-specific concentration and non-concentration parameters identified for sample QC evaluation, along with their associated thresholds and flagging protocols ('Pass', 'Check', 'Warn', 'Fail') are maintained and updated in the LIMS database, and sample QC assessment is performed and documented as part of the run review process. A sample QC result flagged as 'Fail' should not be reported. A sample QC results flagged 'Warn' or 'Check' should be reviewed both by the analyst and supervisor to determine if the quality of the result is suitable for reporting. Results that are flagged during sample QC evaluation may also be assigned one of a series of standard comment codes available in the LIMS database to identify the nature of the sample QC flag.

For details on how the sample QC criteria are used, see "**JA-4027-DR-03-STARLIMS Data Review and Criteria" in Appendix B, section D**. At a minimum, the following parameters are subject to sample QC evaluation in this method. Additional parameters may also be included as needed:

- Calibration curve R^2 is ≤0.95 ('Fail', run is repeated); >0.95 - ≤0.98 ('Check'; Requires Approval)
- Final result is <LOD ('Warn', result is reviewed to determine whether it is code 37 or sample analysis needs to be repeated)
- Diluted instrument result is <LOD ('Fail', results is coded no reportable (code 97)
- Instrument result is >highest calibrator ('Fail', sample analysis is repeated with diluted sample)
- Relative retention time (retention time quantitation ion/retention time ISTD) ≥0.98 - ≤1.02 ('Check', after checking the chromatography, decide whether repeat analysis is needed)
- Confirmation ion ratio (confirmation ion area/quantitation ion area) is outside prespecified analyte ranges (see "**JA-4027-DR-03-STARLIMS Data Review and Criteria" in Appendix B, section D**) ('Check', review chromatography and repeat for all retest 0 samples)
- Concentration ratio (confirmation ion concentration/quantitation ion concentration) is ≥0.7 - ≤1.3 ('Check', review chromatography and repeat if >LOD)
- Both, confirmation ion ratio and concentration ratio are outside prespecified ranges ('Fail', repeat analysis
- Percent difference of Individual ISTD area from within-run average is >20% ('Check', repeat analysis)
- 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

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

For initial steps to investigate QC failures, refer to **"JA-4027-DR-05-Out-of-control Corrective Action" in Appendix B, section D**. The following steps are provided as a general guideline for identifying possible problems resulting in "out of control" values for QC materials. The troubleshooting process should be done in consultation with the supervisor and may involve additional experiments beyond what is indicated below.

(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. Check mass calibration with calibration solution or call for service 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 and/or inaccuracy are pipetting errors. Other sources of procedural errors 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 F**. Refer to the latest *DLS Policies and Procedures Manual* for further information on ruggedness testing. **Appendix E** 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 ambient temperature (+15°C to +30°C) before aliquoting for testing, and then be promptly refrozen for storage (-50°C to -90°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 25OHD$_3$. Thus, the current LC-MS/MS method is preferred. The analyst should refrigerate all processed specimens (+2°C to +8°C) for up to two weeks or keep them deep frozen (-50°C to -90°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, data are transmitted electronically on a regular basis (approximately weekly for certain rapid turnaround analytes), or at the end of a survey cycle. 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. 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

This protocol does not involve referral of specimens for testing the analytes of this method at another laboratory.

A STARLIMS 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 deep frozen (-50^{o}C to -90^{o}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 STARLIMS database.

19. SUMMARY STATISTICS AND GRAPHS

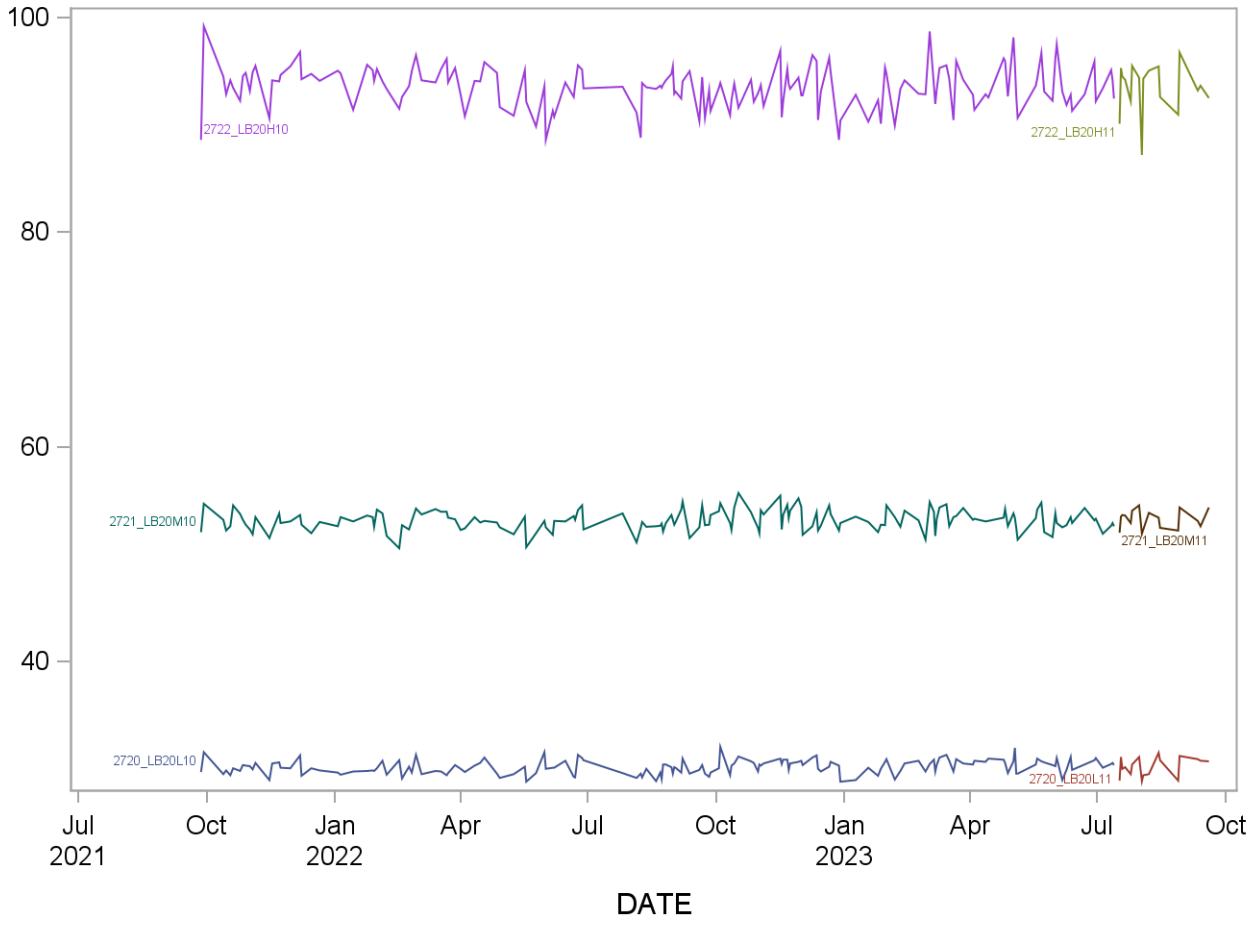
Please see following pages.

August 2021 – August 2023 Summary Statistics and QC Chart
LBXVD2S (25OHD2 (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2722_LB21H10	162	27SEP21	13JUL23	20.266	0.664	3.3
2720_LB21L10	162	27SEP21	13JUL23	4.839	0.190	3.9
2721_LB21M10	162	27SEP21	13JUL23	10.253	0.350	3.4
2722_LB21H11	17	17JUL23	19SEP23	20.203	0.634	3.1
2720_LB21L11	17	17JUL23	19SEP23	4.803	0.220	4.6
2721_LB21M11	17	17JUL23	19SEP23	10.089	0.288	2.9

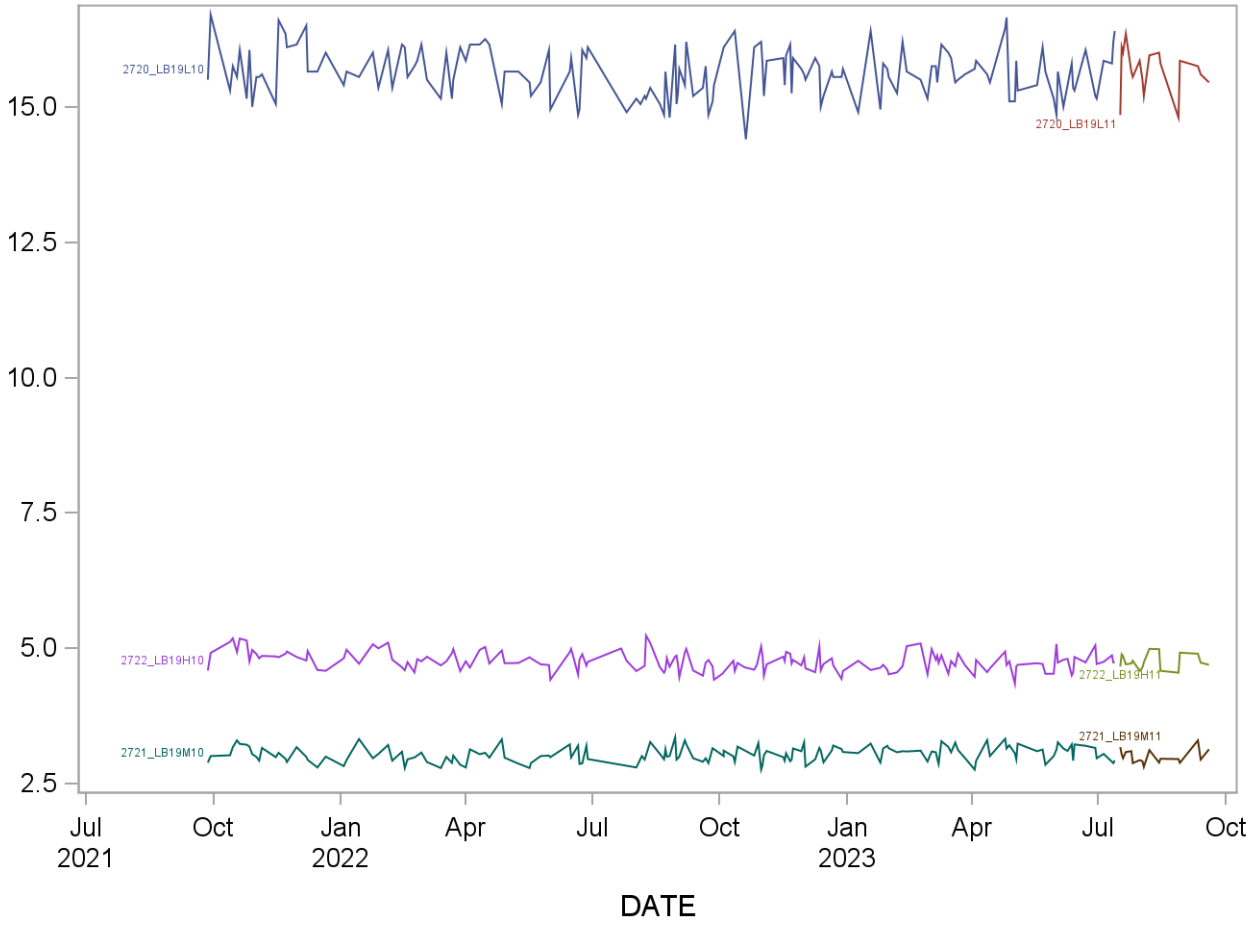
August 2021 – August 2023 Summary Statistics and QC Chart
LBXVD3S (25OHD3 (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2722_LB20H10	163	27SEP21	13JUL23	93.532	1.948	2.1
2720_LB20L10	162	27SEP21	13JUL23	30.174	0.656	2.2
2721_LB20M10	163	27SEP21	13JUL23	53.133	0.937	1.8
2722_LB20H11	17	17JUL23	19SEP23	93.403	2.347	2.5
2720_LB20L11	17	17JUL23	19SEP23	30.215	0.888	2.9
2721_LB20M11	16	17JUL23	19SEP23	53.278	0.863	1.6



August 2021 – August 2023 Summary Statistics and QC Chart
LBXVE3S (epi-25OHD3 (nmol/L))

Lot	n	Start Date	End Date	mean	Standard Deviation	Coefficient of Variation
2722_LB19H10	160	27SEP21	13JUL23	4.764	0.176	3.7
2720_LB19L10	158	27SEP21	13JUL23	15.624	0.443	2.8
2721_LB19M10	157	27SEP21	13JUL23	3.043	0.140	4.6
2722_LB19H11	17	17JUL23	19SEP23	4.761	0.140	2.9
2720_LB19L11	17	17JUL23	19SEP23	15.662	0.417	2.7
2721_LB19M11	17	17JUL23	19SEP23	3.006	0.129	4.3



20. 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 approval of this procedure by the Branch Chief and CLIA Director denotes that the method performance is fit for the intended use of the method.**

References

(1) Olkowski AA, Aranda-Osorio G, and McKinnon J. Rapid HPLC method for measurement of vitamin D3 and 25(OH)D3 in blood plasma. Int J Vitam Nutr Res,.2003;73(1):15-18.

(2) Saenger AK, Laha TJ, Bremner DE, and Sadrzadh SMH. Quantification of serum 25-hydroxyvitamin D2 and D3 using HPLC-tandem mass spectrometry and examination of reference intervals for diagnosis of vitamin D deficiency. Am J Clin Pathol, 2006;125:914-920.

(3) Singh RJ, Taylor RL, Reddy GS, Grebe SK. C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. J Clin Endocrinol Metab 2006;91:3055-61.

(4) Holick MF. The influence of vitamin D on bone health across the life cycle. J Nutr, 2005;135:2726S-2727S.

(5) Holick MF. The vitamin D epidemic and its health consequences. J Nutr 2005:2739S-2748S.

(6) Al-Oanzi ZH, Tuck SP, Harrop JS, Summers GD, Cook DB, Francis RM, and Datta HK. Assessment of vitamin D status in male osteoporosis. Clin Chem, 2006;52(2):248-254.

(7) Nakamura K, Nashimoto M, Hori Y, and Yamamoto M. Serum 25-hydroxyvitamin D concentrations and related dietary factors in peri- and postmenopausal Japanese women. Am J Clin Nutr, 2000;71:1611-1615

(8) Schleicher RL, Encisco S, Chaudhary-Webb M, Paliakov E, McCoy LF, Pfeiffer CM. Isotope-dilution ultra performance liquid chromatography-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 in human serum. Clin Chim Acta 2011 Aug;412(17-18):1594-1599

(9) U.S. Centers for Disease Control and Prevention. National Report on Biochemical Indicators of Diet and Nutrition in the U. S. Population 1999-2002. Atlanta (GA): CDC; 2008. http://www.cdc.gov/nutritionreport/99-02/

(10) Caudill SP, Schleicher RL, Pirkle JL. 2008. Multi-rule quality control for the age-related eye disease study. Stat Med 27:4094-4106.

(11) Schleicher RL, Sternberg MR, Looker AC, Yetley EA, Lacher DA, Sempos CT, Taylor CL, Durazo-Arvizu RA, Maw KL, Chaudhary-Webb M, Johnson CL, Pfeiffer CM. National estimates of serum total 25-hydroxyvitamin D and metabolite concentrations measured by liquid chromatography-tandem mass mpectrometry in the US population during 2007-2010. J Nutr, 2016; 146:1051-61.

(12) IOM (Institute of Medicine). 2011. Dietary reference intakes for calcium and vitamin D. Washington, DC: The National Academies Press.

(13) Landin-Wilhelmsen K, Wilhelmsen L, Wilske J, Lappas G, Rosen T, Lindstedt G, Lundberg PA, and Bengtsson BA. Sunlight increases serum 25(OH) vitamin D concentration whereas 1,25(OH)2D3 is unaffected. Results from a general population study in Goteborg, Sweden (The WHO MONICA Project). Eur J Clin Nutr, 1995 June;49(6):400-407.

(14) Hine TJ, Roberts NB. Seasonal variation in serum 25-hydroxy vitamin D3 does not affect 1,25-dihydroxy vitamin D. Ann Clin Biochem, 1994 Jan.;31(1):31-34.

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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**	75.1	69.0	72.7	74.0	73.4	74.1	2.15	2.9	3.2
	2		75.9	75.9	74.3	76.5	74.0				
Level 2	1	**45.1**	46.2	45.1	42.1	46.2	47.0	45.7	1.54	3.4	1.4
	2		46.7	46.0	45.1	45.1	47.7				
Level 3	1	**49.5**	51.4	49.5	49.5	48.6	50.4	50.4	0.97	1.9	1.7
	2		51.6	50.3	50.1	50.9	51.3				
Level 4	1	**73.4**	75.0	72.5	73.7	76.9	74.8	74.8	1.64	2.2	1.9
	2		76.3	76.0	72.3	76.4	74.0				

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*	1.64	0.805	1.29	1.30	0.752	1.09	0.27	25.2	-16.2
	2		0.858	1.21	1.05	1.06	0.929				
Level 2	1	**2.00**	2.17	2.03	1.97	1.78	1.77	1.88	0.20	10.4	-6.0
	2		1.98	1.53	2.02	1.65	1.91				
Level 3	1	**32.0**	34.0	35.3	33.3	32.9	32.8	33.7	0.86	2.5	5.4
	2		34.4	34.1	33.6	32.6	34.4				
Level 4	1	*1.30*	0.961	1.36	1.17	0.870	1.47	1.18	0.23	19.8	-9.5
	2		1.11	1.58	1.23	0.973	1.04				

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**	5.19	4.70	4.36	4.88	4.96	4.80	0.24	5.0	6.7
	2		4.83	4.61	4.60	5.00	4.88				
Level 2	1	**3.20**	3.30	3.49	3.25	3.03	3.49	3.27	0.14	4.2	2.3
	2		3.21	3.31	3.18	3.24	3.22				
Level 3	1	*2.90*	2.94	2.89	3.00	2.65	2.72	2.86	0.14	4.9	-1.4
	2		2.76	2.88	2.71	3.02	3.02				
Level 4	1	**64.8**	65.0	59.3	63.0	63.9	68.7	63.8	3.10	4.9	-1.5
	2		63.6	61.6	62.0	62.1	69.2				

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 001-005**
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	40.6	39.4	40.6	38.4	39.0	39.9	39.7	0.88	2.2	-2.4
	2		40.1	40.2	38.1	40.4	40.4				
Level 2	1	23.8	23.9	23.6	24.5	24.0	23.1	23.8	1.00	4.2	0.1
	2		26.1	23.6	24.1	22.4	23.1				
Level 3	1	29.3	29.0	30.5	31.2	30.2	29.7	30.4	0.71	2.3	3.6
	2		30.2	30.7	30.6	31.5	30.5				
Level 4	1	16.5	16.7	17.3	14.7	16.6	17.3	16.6	0.75	4.5	1.2
	2		17.0	16.4	16.5	17.1	16.8				
Level 5	1	71.7	73.8	70.2	82.4	75.7	74.2	74.8	3.36	4.5	4.4
	2		77.5	74.0	72.8	72.0	75.7				

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 001-005**
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	2.25	2.76	3.36	3.13	2.92	2.86	0.39	13.8	-5.0
	2		3.29	2.24	2.79	2.71	3.16				
Level 2	1	3.08	3.07	2.72	2.60	3.73	2.80	2.76	0.50	17.9	-10.3
	2		1.91	2.62	2.21	2.98	2.99				
Level 3	1	13.2	14.4	13.7	14.4	13.3	13.8	14.0	0.39	2.8	6.4
	2		14.6	13.7	14.1	14.0	14.0				
Level 4	1	16.7	18.8	15.7	16.7	16.2	18.2	17.3	1.02	5.9	3.0
	2		18.1	17.0	16.4	17.3	18.1				
Level 5	1	n/a	0.147	0.799	1.00	1.60	1.30	0.959	0.43	45.3	
	2		1.54	0.973	0.675	0.774	0.780				

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 001-005**
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	2.17	1.19	2.24	2.09	2.05	2.26	2.05	0.33	16.0	-5.4
	2		1.95	2.37	2.18	2.02	2.17				
Level 2	1	<LOQ	0.975	0.392	1.12	1.82	1.14	1.06	0.38	36.0	
	2		1.11	0.593	1.09	1.28	1.09				
Level 3	1	1.57	1.12	1.43	1.47	1.41	1.69	1.38	0.23	16.5	-12.4
	2		1.14	1.54	1.31	1.01	1.63				
Level 4	1	<LOQ	0.894	0.724	1.28	1.83	1.10	1.04	0.43	41.0	
	2		1.45	0.304	0.856	0.780	1.15				
Level 5	1	4.19	3.50	4.01	4.28	4.23	4.10	3.89	0.30	7.7	-7.2
	2		3.97	3.98	3.46	3.52	3.84				

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	29.0	30.4	29.70	0.4900	0.4900	1764.1800
2	30.7	30.5	30.60	0.0100	0.0100	1872.7200
3	30.3	30.3	30.30	0.0000	0.0000	1836.1800
4	30.0	32.3	31.15	1.3225	1.3225	1940.6450
5	29.1	30.6	29.85	0.5625	0.5625	1782.0450
6	30.9	30.3	30.60	0.0900	0.0900	1872.7200
7	28.7	31.3	30.00	1.6900	1.6900	1800.0000
8	29.3	29.7	29.50	0.0400	0.0400	1740.5000
9	29.0	31.7	30.35	1.8225	1.8225	1842.2450
10	30.8	31.0	30.90	0.0100	0.0100	1909.6200

Grand sum	605.9	**Grand mean**	30.295

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	12.0750	1.2075	1.098863049	3.63
Between Run	5.1145	0.568277778	0	0.00
Total	17.1895		1.098863049	**3.63**

Quality material 2 (HS12475)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	95.0	95.0	95.00	0.0000	0.0000	18050.0000
2	90.2	92.8	91.50	1.6900	1.6900	16744.5000
3	93.2	93.5	93.35	0.0225	0.0225	17428.4450
4	96.4	98.8	97.60	1.4400	1.4400	19051.5200
5	88.5	93.7	91.10	6.7600	6.7600	16598.4200
6	96.6	93.7	95.15	2.1025	2.1025	18107.0450
7	98.3	93.6	95.95	5.5225	5.5225	18412.8050
8	93.2	87.1	90.15	9.3025	9.3025	16254.0450
9	90.4	93.5	91.95	2.4025	2.4025	16909.6050
10	93.2	93.0	93.10	0.0100	0.0100	17335.2200

Grand sum	1869.7	**Grand mean**	93.485

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	58.505	5.8505	2.418780685	2.59
Between Run	102.7005	11.41116667	1.667433157	1.78
Total	161.2055		2.937827996	**3.14**

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	4.61	5.23	4.92	0.0961	0.0961	48.4128
2	4.89	5.02	4.96	0.0042	0.0042	49.1041
3	5.13	4.91	5.02	0.0121	0.0121	50.4008
4	4.47	4.94	4.71	0.0552	0.0552	44.2741
5	5.04	5.00	5.02	0.0004	0.0004	50.4008
6	4.85	4.81	4.83	0.0004	0.0004	46.6578
7	4.83	4.51	4.67	0.0256	0.0256	43.6178
8	4.76	5.26	5.01	0.0625	0.0625	50.2002
9	4.74	4.63	4.69	0.0030	0.0030	43.8985
10	5.03	4.94	4.99	0.0020	0.0020	49.7005
Grand sum	97.6	**Grand mean**	4.88			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.5232	0.05232	0.228735655	4.69
Between Run	0.3792	0.042133333	0	0.00
Total	0.9024		0.228735655	**4.69**

Quality material 2 (HS12475)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	20.6	20.9	20.75	0.0225	0.0225	861.1250
2	20.3	20.4	20.35	0.0025	0.0025	828.2450
3	19.9	21.0	20.45	0.3025	0.3025	836.4050
4	20.0	21.5	20.75	0.5625	0.5625	861.1250
5	19.9	20.8	20.35	0.2025	0.2025	828.2450
6	20.3	20.3	20.30	0.0000	0.0000	824.1800
7	17.4	19.9	18.65	1.5625	1.5625	695.6450
8	20.0	21.1	20.55	0.3025	0.3025	844.6050
9	18.9	21.0	19.95	1.1025	1.1025	796.0050
10	20.2	20.0	20.10	0.0100	0.0100	808.0200
Grand sum	404.4	**Grand mean**	20.22			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	8.14	0.814	0.902219485	4.46
Between Run	6.632	0.736888889	0	0.00
Total	14.772		0.902219485	**4.46**

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	15.2	16.2	15.70	0.2500	0.2500	492.9800
2	15.8	16.0	15.90	0.0100	0.0100	505.6200
3	16.0	15.9	15.95	0.0025	0.0025	508.8050
4	14.5	15.6	15.05	0.3025	0.3025	453.0050
5	15.1	15.4	15.25	0.0225	0.0225	465.1250
6	15.8	16.5	16.15	0.1225	0.1225	521.6450
7	16.6	16.9	16.75	0.0225	0.0225	561.1250
8	16.8	14.9	15.85	0.9025	0.9025	502.4450
9	15.8	15.9	15.85	0.0025	0.0025	502.4450
10	15.7	15.4	15.55	0.0225	0.0225	483.6050

Grand sum	316	**Grand mean**	15.8

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	3.32	0.332	0.576194412	3.65
Between Run	4	0.444444444	0.237112257	1.50
Total	7.32		0.623074813	**3.94**

Quality material 2 (HS12475)

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1	4.83	4.96	4.90	0.0042	0.0042	47.9221
2	4.67	4.82	4.75	0.0056	0.0056	45.0301
3	4.96	5.06	5.01	0.0025	0.0025	50.2002
4	4.87	4.27	4.57	0.0900	0.0900	41.7698
5	4.58	4.93	4.76	0.0306	0.0306	45.2201
6	4.83	5.05	4.94	0.0121	0.0121	48.8072
7	5.78	4.43	5.11	0.4556	0.4556	52.1221
8	4.54	4.29	4.42	0.0156	0.0156	38.9845
9	4.59	5.21	4.90	0.0961	0.0961	48.0200
10	4.95	4.96	4.96	0.0000	0.0000	49.1041

Grand sum	96.58	**Grand mean**	4.829

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev
Within Run	1.4249	0.14249	0.377478476	7.82
Between Run	0.79508	0.088342222	0	0.00
Total	2.21998		0.377478476	**7.82**

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: 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: 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: 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: samples stored at -80°C for 7 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**	
	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	30.3
Replicate 2	28.3	28.7	28.3	31.3	29.1	29.5	29.7	30.6
Replicate 3	29.9	27.6	29.9	30.6	33.9	30.6	29.6	30.5
Mean	29.2	28.2	29.2	30.5	31.3	29.8	29.8	30.5
% difference from initial measurement	--	**-3.53**	--	**4.33**	--	**-4.90**	--	**2.38**

Quality material 2							**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	93.6
Replicate 2	83.8	86.3	83.8	86.9	84.6	87.9	92.9	93.3
Replicate 3	87.9	87.7	87.9	87.5	86.7	90.2	96.9	93.5
Mean	86.1	87.0	86.1	88.8	85.9	87.0	95.5	93.5
% difference from initial measurement	--	**1.05**	--	**3.14**	--	**1.33**	--	**-2.08**

In August 2021, LC-MS/MS System TSQ Vantage was replaced with TSQ Altis, and the isocratic HPLC method was replaced with gradient HPLC method. However, these changes did not impact the sample preparation and thus stability experiments were not conducted. We collected an additional data point for long-term stability with the current method (7 years).

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: 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: 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: 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: samples stored at -80°C for 7 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**	
	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	4.68
Replicate 2	4.79	4.61	4.79	5.04	70.6	67.3	4.31	4.77
Replicate 3	4.15	4.28	4.15	4.92	70.3	65.1	5.76	4.51
Mean	4.66	4.55	4.66	4.90	71.5	67.3	5.20	4.65
% difference from initial measurement	--	**-2.29**	--	**5.22**	--	**-5.82**	--	**-10.5**

Quality material 2							**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	21.5
Replicate 2	18.3	19.9	18.3	18.9	45.8	39.3	22.4	21.7
Replicate 3	17.8	19.1	17.8	18.9	43.6	40.9	22.5	21.4
Mean	18.4	19.4	18.4	19.3	44.9	40.5	21.8	21.5
% difference from initial measurement	--	**5.44**	--	**4.90**	--	**-9.77**	--	**-1.19**

In August 2021, LC-MS/MS System TSQ Vantage was replaced with TSQ Altis, and the isocratic HPLC method was replaced with gradient HPLC method. However, these changes did not impact the sample preparation and thus stability experiments were not conducted. We collected an additional data point for long-term stability with the current method (7 years).

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: 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: 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: 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: samples stored at -80°C for 7 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	
	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	15.5
Replicate 2	14.7	15.0	14.7	16.2	47.1	41.8	16.7	16.1
Replicate 3	16.8	14.2	16.8	15.2	47.6	45.5	15.1	15.6
Mean	15.9	15.0	15.9	15.5	46.2	44.3	15.7	15.7
% difference from initial measurement	--	**-5.86**	--	**-2.72**	--	**-4.26**	--	**0.16**

Quality material 2							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	4.91
Replicate 2	4.40	5.02	4.40	5.10	19.4	19.5	5.55	4.95
Replicate 3	4.89	5.03	4.89	5.00	22.5	18.4	6.08	5.08
Mean	4.95	5.03	4.95	4.92	20.5	19.0	5.45	4.98
% difference from initial measurement	--	**1.68**	--	**-0.61**	--	**-7.46**	--	**-8.60**

In August 2021, LC-MS/MS System TSQ Vantage was replaced with TSQ Altis, and the isocratic HPLC method was replaced with gradient HPLC method. However, these changes did not impact the sample preparation and thus stability experiments were not conducted. We collected an additional data point for long-term stability with the current method (7 years).

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	2.216	yes	yes
25(OH)D3	0.439	yes	yes
C3-epi-25(OH)D3	0.755	yes	yes

References for LOD Determination in Appendix A:

1. Taylor JK. Quality Assurance of Chemical Measurements. Boca Raton: Lewis Publishers (CRC Press); 1987.

2. Protocols for determination of limits of detection and limits of quantitation; approved guideline. volume 24, no. 34. EP17-A. 2004.

Appendix B: Job Aids

A. General

1) JA-4027-G-01-Calibration and Calibration Verification

(a) Calibration

– Assay Calibration

Generally, this assay is calibrated **daily** using a 7-point calibration curve (each levels in duplicate) with an alternate high calibrator to conduct repeat analysis when specimens exceed cal7. Typical calibration curves are shown in the table below (nmol/L):

Analyte	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	Cal 8
25-hydroxyvitamin D2 (VID2)	1.07	5.05	9.80	18.0	34.1	48.3	65.0	112.9
25-hydroxyvitamin D3 (VID3)	6.06	12.9	36.7	56.9	96.2	132	174	291.9
C3-epimer-25-hydroxyvitamin D3 (VID3E)	0.896	4.10	7.33	13.1	24.8	35.2	47.1	72.6

The sensitivity of this assay is defined in the table below (nmol/L):

Sensitivity	VID2 * (nmol/L)	VID3 ** (nmol/L)	VID3E *** (nmol/L)
LOD †	2.216	0.439	0.755

* Low QC pool diluted to 0.3-1.7 nmol/L (3 days in triplicate measurements)
** Low QC pool diluted to 0.9-6.2 nmol/L (3 days in triplicate measurements)
*** Low QC pool diluted to 0.6-3.3 nmol/L (3 days in triplicate measurements)
† LOD (Limit of Detection) is based on a 2.5% probability that a result at LOD is a false positive

Confirmation of Results and Reportable Range
Samples are re-tested to verify results when concentrations are as follows (for example):

VID2 >65.0 nmol/L
VID3 <12.5 nmol/L or >174 nmol/L
VID3E >47.1 nmol/L

Historically, total VID <12.5 nmol/L were always confirmed. No retest criteria for low levels have been established for VID2 and VID3E because many-to-most results are <LOD.
The reportable range (analytical measurement range) of concentrations without dilution is from the **LOD to high calibration point (nmol/L)**. However, method validation demonstrated this method to be linear to *at least* twice these levels. In mid-2015, this method was amended (see DLS #4027.05) to permit the use of cal8 for repeat analysis on specimens that exceed cal7 without dilution.

(b) Calibration Verification

– Assay Calibration Verification

According to the CLIA regulations from 2003, the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration materials, and includes low, mid, and high value, and is performed at least once every six months. All these conditions are met with the calibration of this assay (it generally uses 7 calibration points, which are also duplicated), and therefore no additional calibration verification is required by CLIA.

Acceptable Calibration Verification Materials:
Currently available NIST 25-hydroxyvitamin D Standard Reference Materials (SRM) are:

a) SRM 972 [superseded by SRM 972a]
b) SRM 972a [Matrix: Serum; Analytes: VID2, VID3 and VID3E; 4 levels per each analyte]
c) SRM 2972 [superseded by SRM 2972a]
d) SRM 2972a [Matrix: Ethanol; Analytes: VID2 (1 level), VID3 (2 Levels) and VID3E (1 level)]
e) SRM 2973 [Matrix: Serum; Analytes: VID2, VID3 and VID3E; 1 level]

The certified concentrations, unless specified otherwise, of vitamin D metabolites in SRM 972a, SRM 2972a and SRM 2973 are provided by National Institute of Standards and Technology (NIST, Gaithersburg, MD) as follows (± expanded uncertainty):

Standard Reference Materials	VID2 (nmol/L)	VID3 (nmol/L)	VID3E (nmol/L)
SRM 972a Level 1	1.3 ± 0.2*	71.8 ± 2.7	4.5 ± 0.2
SRM 972a Level 2	2.0 ± 0.2	45.1 ± 1.0	3.2 ± 0.2
SRM 972a Level 3	32.0 ± 0.8	49.5 ± 1.1	2.9 ± 0.4*
SRM 972a Level 4	1.3 ± 0.2*	73.4 ± 2.3	64.8 ± 5.4
SRM 2972a VID3 Level 1	NA	806.2 ± 32.4	NA
SRM 2972a VID3 Level 2	NA	1596.5 ± 64.1	NA
SRM 2972a VID2	560.4 ± 19.9	NA	NA
SRM 2972a VID3E	NA	NA	577.0 ± 28.5
SRM 2973	1.59 ± 0.05*	98.4 ± 2.1	5.23 ± 0.20*

*Reference Values; NA: not applicable

Note: To convert from nmol/L to ng/mL, use the following equation:

nmol/L = ng/mL x conversion factor

Conversion factors obtained from NIST certificates are 2.4234 for 25(OH)D2 and 2.4960 for 25(OH)D3 and 3-epi-25(OH)D3.

The CDC lab verifies calibration across the entire range. Calibration verification may be conducted by the analysis of NIST standard reference materials (SRM) in conjunction with the calibrators to be verified or testing the calibrators as unknowns by the Reference Measurement Procedure (RMP) DLS method #4029.

To verify the calibration across the reportable range (analytical measurement range), one or more of the following are used:

1) independently prepared calibration materials
2) SRM 972 and/or 972a
3) diluted SRM 2972 and/or 2972a
4) SRM 2973
5) testing of the full range of calibrators by reference method 4029

Every time a new lot of calibration solutions are prepared for method #4027, verifying materials (at least three levels spanning the calibration range) are prepared for analysis using method #4027. More recently, method #4029 is being used to value-assign the concentrations of the new lot of calibrators.

For calibrators that are at least 10 nmol/L, average results from 2-3 runs for VID2 and VID3 calibrators are expected to be ± 15% of the target values. In the event that the calibrators do not meet specifications, SRM materials are the gold standard for adjusting in-house calibrators. Either SRM 972, SRM 972a, SRM 2972, SRM 2972a or SRM 2973 may be used for this purpose.

Furthermore, method #4027 analyzes serum samples provided by method #4029 throughout the year and verifies the VID2 and VID3 results as needed against those of method #4029 as a form of calibration verification.

Additionally, the master stock solutions that were used to prepare calibrators were reassessed annually until 2015 and as needed thereafter on the UV-Vis spectrophotometer to confirm the accuracy of the assigned concentrations for the master stock. Periodic concentration reassignments may be applied from this assessment.

B. Reagents & Standards

1) JA-4027-R&S-01-Reagents & Standards Tracking

Tracking chemicals and other materials used in the preparation of reagents, calibrators, and quality control materials

For details on labeling requirements, see the Division of Laboratory Sciences Safety and Quality Joint Labeling Requirements.

(a) Reagent Preparation

– Frequently Prepared Reagents

The following reagents specified in the APM are considered to be “frequently-prepared reagents”

- HPLC Mobile Phase Line 1 (72% methanol, 28% water)
- HPLC Mobile Phase Line 2 (100% methanol)
- HPLC Mobile Phase Line 3 (100% water)
- HPLC Needle Wash (72% methanol, 28% water)
- HPLC Seal Wash (10% methanol, 90% water)
- Extraction Solvent (72% methanol, 28% water)
- Extraction Solvent (100% hexane)

Information available on the chemicals used to prepare the “frequently-prepared reagents” identified above is maintained on reagent tracking sheets in the laboratory.

– Infrequently Prepared Reagents

The following reagents specified in the APM are considered to be “infrequently-prepared reagents” for this SOP:

- 0.01M Phosphate-buffered saline (PBS); pH 7.4
- 4% Albumin in 0.01M PBS
- Mass Spectrometer Equilibration Test Solution (72% methanol, 28% water)

Information available on chemicals used to prepare the “infrequently-prepared reagent” identified above is tracked in individual records (either written or electronic) generated when the reagent is prepared.

(b) Standards Preparation

– Stock Solutions

All calibrators and internal standards specified in the APM originate from single-analyte stock solutions. Information available on chemicals and other materials (e.g., low-baseline human serum samples, 100% ethanol) used to prepare stock solutions is tracked in records generated when the stock solution is prepared.

- Working Standard Solutions

  Stock solutions are mixed as specified in the APM to create mixed-analyte working standard solutions. Information available on the stock solutions used to prepare the working standard solutions is tracked in individual records (either written or electronic) generated when the working standard is prepared.

(c) Quality Control Materials

Chemicals and other materials (e.g., human serum samples) used to prepare quality control materials are tracked in individual records (either written or electronic) generated when the material is prepared.

Chemicals may be used to amend (i.e., spike) analyte into a quality control material. In these cases, available information on the chemicals used is tracked in the record.

Human serum samples may be used to prepare quality control materials. In these cases, available information on the human serum samples used is tracked in the record.

(d) Other Materials

To facilitate tracking of solvents and other chemicals transferred from the original manufacturer containment into a secondary container (e.g., solvent bottle), include the expiration date provided by the manufacturer or the lot number on the secondary container.

C. Instruments

1) JA-4027-I-01-LC-MS/MS Instrument Comparison and System Verification

1. Instrument Comparison

When a method is analyzed on multiple instruments, an initial instrument comparison must be conducted to establish analytical comparability. All calibrators, quality control materials, and at least 30 samples that span the measurement range should be analyzed on each system. The same preparation should be analyzed on all systems on the same day. However, this may not be feasible. In this case, it is acceptable to prepare different aliquots of the samples and perform the analysis as close to the same time as possible. The results from each instrument should be plotted against the results from the original instrument. The parameters assessed are correlation (Pearson r >0.95), regression fit (r^2), and slope (m). The same data analysis and documentation procedure as described below under System Verification can be used.

2. System Verification

Following the initial instrument comparison, semi-annual system verification is performed to ensure that the systems are maintaining comparability.

Requirement: According to DLS Policies and Procedures (section 12.2), if a DLS method is run on multiple instruments or at multiple sites, a set of at least five samples spanning the reportable range of the analytes must be run at least once every six months. The Pearson correlation coefficient of the compared results should be greater than 0.95, and if not, appropriate corrective action should be taken. In special situations, the Division Director may give written approval that the methods are sufficiently similar for the intended use of the data.

Procedure: No separate sample preparation is needed. A set of samples spanning approximately the reportable range that were analyzed on the primary instrument, are re-analyzed shortly on the secondary instrument. The time delay should be within the processed sample stability parameters determined during method validation (e.g., sample kept in autosampler or refrigerated/frozen for a certain time).

Data analysis:

- Identify a subset of results (*n* ≥5) from the two analyses and describe any inclusion or exclusion criteria applied (e.g., only include samples with analyte results ≥3x the LOD and ≤ highest calibrator).
- Determine the Pearson correlation coefficient. *Note:* Pearson correlation is a parametric test that requires normally distributed data. Most nutritional biomarkers show right-skewed analyte concentration distributions benefiting from a log-transformation to yield data that approximates a normal distribution. While Pearson analysis verifies correlation and not concordance, high concordance is expected for instrument comparisons because most critical variables are the same (measurement technique, sample preparation, operator, calibration, etc.) and only 1 variable changed (instrument).
- Optional: Assess Lin's rho coefficient for concordance. Perform regression and Bland-Altman bias analysis. Assess whether a similar proportion of samples is <LOD on

both instruments. *Note:* Most nutritional biomarkers show non-constant variance (constant CV with increasing concentration); thus, weighted Deming regression and relative (%) Bland-Altman analysis are generally preferred.

Documentation, review, and approval:

- Summarize the results in a spreadsheet that contains the raw data, the data analyses, and the summary information and request review by the supervisor. For an example, see: Instrument comparison - Template with data 4063 caffeine.xlsx

- General supervisor reviews the data and, if acceptable, approves the data. Convert summary information tab to a PDF and electronically sign the PDF in the designated field. Add the signed PDF to your electronic QA Manual. For an example, see: Instrument comparison - Example signed PDF 4063 caffeine.pdf

2) JA-4027-I-02-Thermo LC-MS/MS System Operation and Maintenance

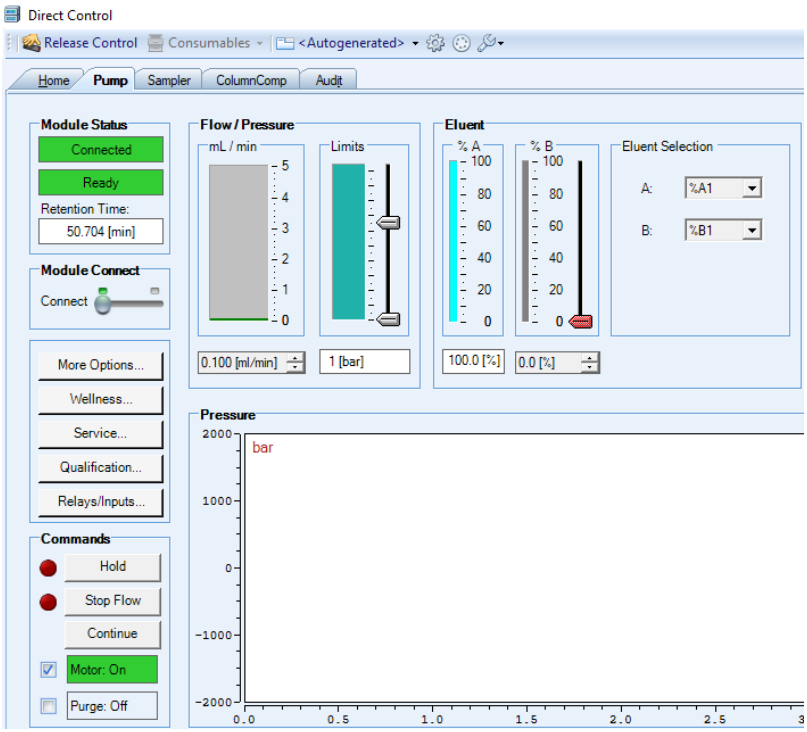
(a) Instrument Preparation and Operation

– Preparation of Solvents

- On Vanquish HPLC system, fill all solvent bottles with appropriate solvents.
  - ◊ Line A1: 72% methanol/ 28% water
  - ◊ Line B1: 100% methanol
  - ◊ Line A2: 100% water
  - ◊ Needle Wash: 72% methanol/ 28% water
  - ◊ Seal Wash: 10% methanol/ 90% water
  - ◊ All other unused lines: 100% MeOH (Use these lines if mixing solvents using pumps)
- To prepare the mobile phase mixture, measure appropriate volume of DI-water using a graduated cylinder. Measure appropriate volume of methanol using a separate graduated cylinder. Place both liquids in a bottle, cap the bottle and shake thoroughly. Pour this mixture into the mobile phase reservoir.
- The composition of the needle wash should match the composition of the initial mobile phase.
- These solutions are stable at ambient temperature (+15^{o}C to +30^{o}C); thus, prepare as needed.

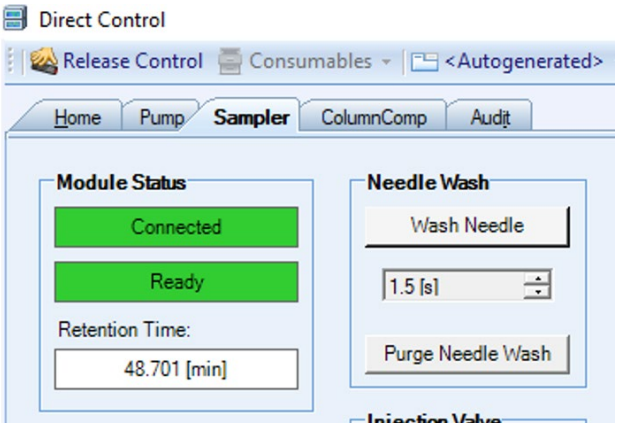
– Purging the Pump

- Purge the solvent lines to remove air bubbles after refilling or replacing solvents.
- To purge the line, open the purge valve (gold knob) on the pump by turning it counterclockwise.
- In Xcalibur window, click on "Thermo Scientific SII for Xcalibur". Click on “Direct Control” button. When the “Direct Control” window opens, click on “Pump” tab.
- Set the channel to purge to 100% by selecting the desired mobile phase line in “Eluent Selection” (%A1, %A2, %A3 and %B1, %B2, %B3) and changing the %B to desired percentage.
- For example, to purge line %A1, select line %A1 and any B lines in the “Eluent Selection” dropdown menu. Then, set the %B indicator to 0%. This will purge %A1 line at 100%. To purge line %B1, select %B1 line and any A lines in the “Eluent Selection” dropdown menu. Then, set the %B indicator to 100%. This will purge %B1 line at 100%.
- Perform the above steps for all channels as needed.
- The standard purge time is usually about 5 minutes per channel at 3mL/min flow rate. This can be adjusted as needed by clicking on “Pump” tab in Direct Control window. Click on “More Options”, then change “Purge Flow and Time”.
- When purging is completed, turn the purge valve clockwise to close the valve.
- Purge valve seals can be damaged if overtightened; thus, only use your fingers to tighten the knob.



– Washing the Needle

- Wash the needle as needed and before a run as a part of a routine maintenance for the needle wash system.
- Wash the needle as needed after replacing needle wash liquid or needle wash lines.
- To flush the needle wash port, open Xcalibur window. Click on "Thermo Scientific SII for Xcalibur". Click on “Direct Control” button. When the “Direct Control” window opens, click on “Sampler” tab. Click on “Wash Needle” button.

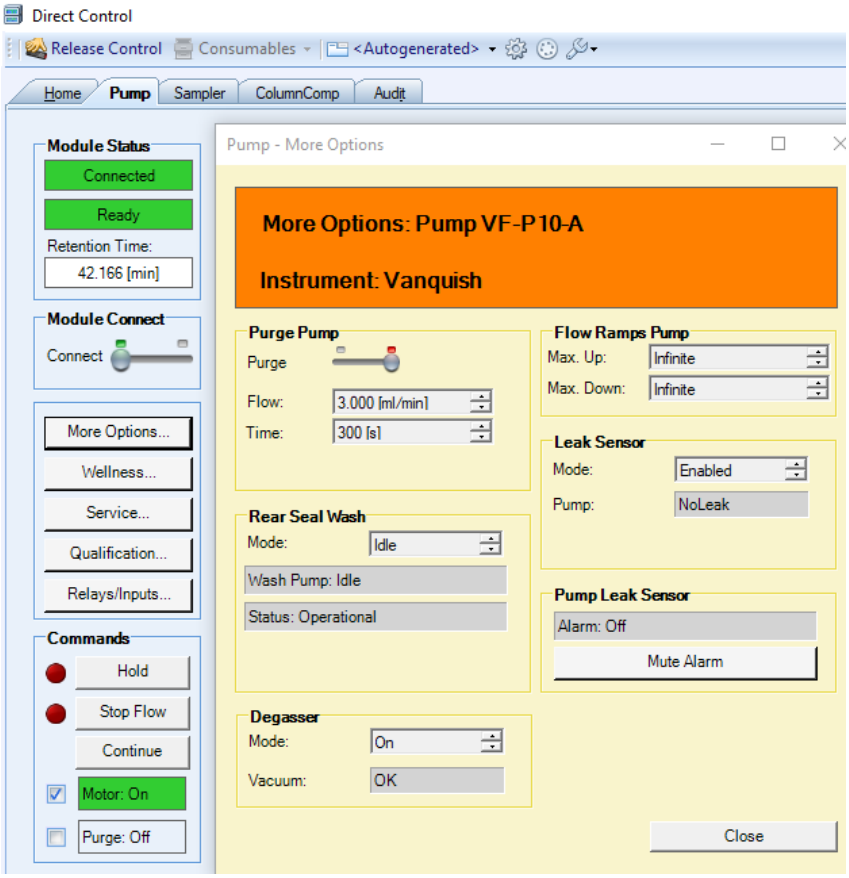


- If replacing the needle wash liquid, click on the “purge needle wash” button to purge the needle wash system with fresh needle wash liquid.
- ***Troubleshooting Tip****: If the wash pump is not responding and continuously running the needle wash after purging,* manually turn the wash pump on and off.

To do so, click on F8 → Right-click, change Level to “Expert” → SamplerModule → Sampler → Properties → Wash Pump → On/Off.

– Check the Seal Wash Status

- Check the seal wash status before starting each run to make sure the status is “Operational”. Open Xcalibur window. Click on "Thermo Scientific SII for Xcalibur”. Click on “Direct Control” button. When the “Direct Control” window opens, click on “Pump” tab. Click on “More Options”.
- Under Rear Seal Wash, check the status.
- If the seal wash status is “Operational”, it is okay to proceed.
- If the seal wash status says “Dry”, change the mode to “Active” to run the seal wash. This will change the status to “Operational” after a few minutes. Then, change the mode back to “Idle”.



- If seal wash status is not operational after these steps, follow the instructions in the instrument manual on testing the seal wash system for leakage using a syringe.

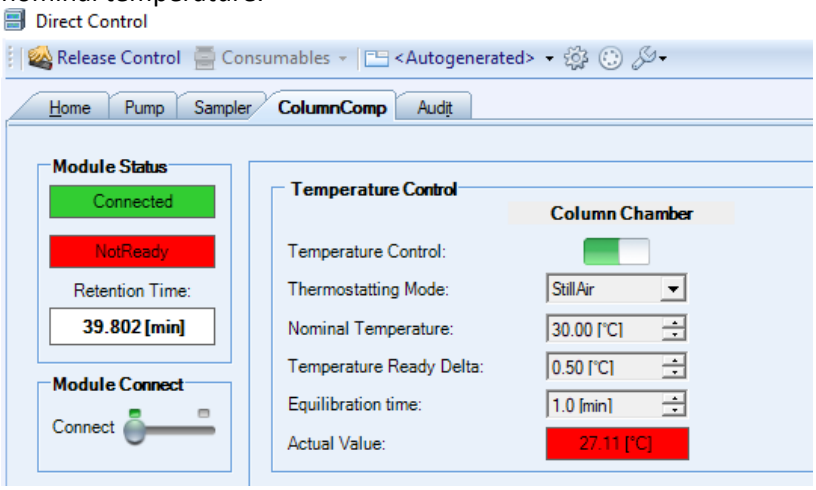
– Checking Autosampler and Pump Settings

- All autosampler and pump settings should be set up as a part of the instrument method file. Generally, the setting for autosampler tray temperature is around 7°C, column temperature is around 30°C and flow rate is around 350 uL/min.

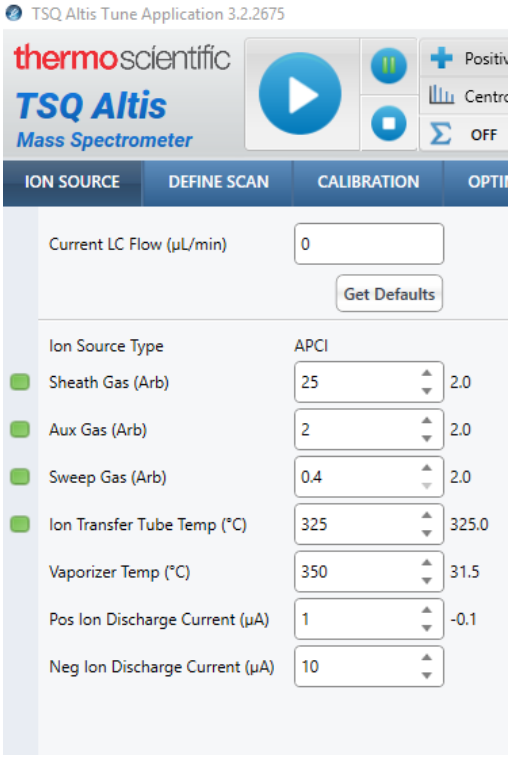
- While run times may differ slightly depending upon column age, each injection generally has the following run parameters if using pre-mixed solvents: **0-8.5 min** [72% methanol:28%water (A) with introduction of 100% methanol (B) until it reaches 80% A to 20% B (Curve: 5)], **8.5-10 min** [100% methanol wash] and **10-13min** [72% methanol:28%water (A)]. If using pump-mixed solvents, similar run parameters are used with methanol and water in separate bottles.

– Getting the Instrument Ready for the Analysis

- Before starting the analysis:
  a. Change the guard column as necessary (if the column pressure is higher than the expected, or peak broadening or excessive tailing is observed).
  b. Change the column as necessary.
  c. Set the autosampler tray to 7°C: Open Xcalibur window. Click on "Thermo Scientific SII for Xcalibur". Click on "Direct Control" button. When the "Direct Control" window opens, click on "Sampler" tab. Set the temperature.
  d. Set the column temperature to 30°C: Open Xcalibur window. Click on "Thermo Scientific SII for Xcalibur". Click on "Direct Control" button. When the "Direct Control" window opens, click on "ColumnComp" tab. Set the nominal temperature.

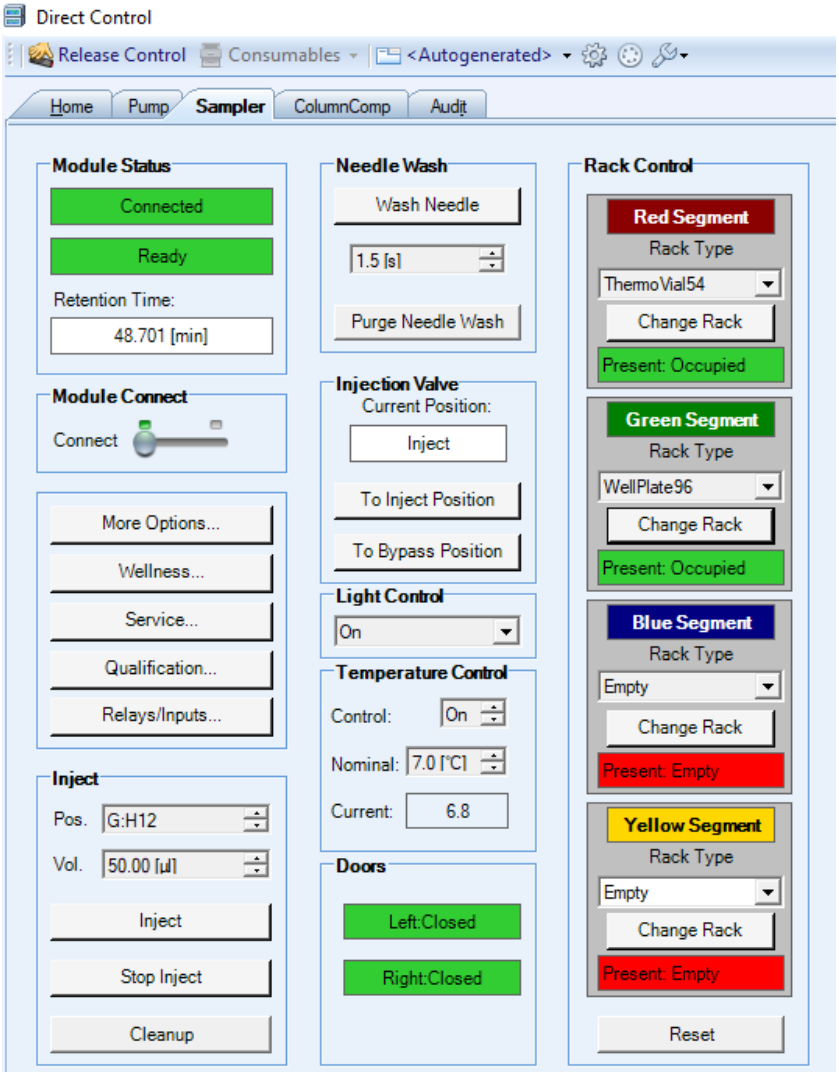


  e. Open "TSQ Altis Tune". Click on "Ion Sources" tab and enter the source parameters to match the parameters in the instrument method file. Turn on the mass spec by clicking the button  . Leave the instrument on for about 30 mins before starting the run and continue to keep it on when starting the run.



f. Equilibrate the column with mobile phase for about 30 minutes. Open Xcalibur window. Click on "Thermo Scientific SII for Xcalibur". Click on "Direct Control" button. When the "Direct Control" window opens, click on "Pump" tab.
    a. Set the "Eluent Selection" to lines %A1 [~72% methanol:28% water] and %B1 [100% methanol]. Then, set the indicator to 0%B to run line %A1 at 100%. If using pump-mixed solvents, set methanol line to ~72% and water line to ~28%.
    b. To protect the ceramic within the APCI source, start with the flow rate of 100uL/min. Check the "Motor: On" box. This will start the mobile phase flow. Then, ramp up the flowrate to 200uL/min, then 300uL/min, and finally ~350uL/min.
    c. Equilibrate the column with ~72% methanol:28% water for about 30 mins at ~350uL/min.
    d. Note the back pressure. Typically, a new column is around 400-450 bar for Ascentis column. The pressure goes up as the column is used. Ensure that the back pressure is not so high that it could pressure out mid-run (runs pressure out when the back pressure reaches 600 bar for Ascentis column). Replace the PFP analytical column as needed based on increased back pressure or poor chromatographic separation.
g. Before starting the run, leave the pump running.
h. Put the plate in the autosampler in desired rack and change the settings to recognize the rack in the autosampler. Open Xcalibur window. Click on "Thermo Scientific SII for Xcalibur". Click on "Direct Control" button. When the "Direct Control" window opens, click on "Sampler" tab. Under "Rack Control", choose the color that represents the rack position (Red, Green,

Blue, Yellow Segment) and Select “WellPlate 96” from the drop-down menu and click on “Change Rack”. If the autosampler door is open, the racks are rotated; thus, this step needs to be repeated to set the plate position again so that the sequence can recognize it.



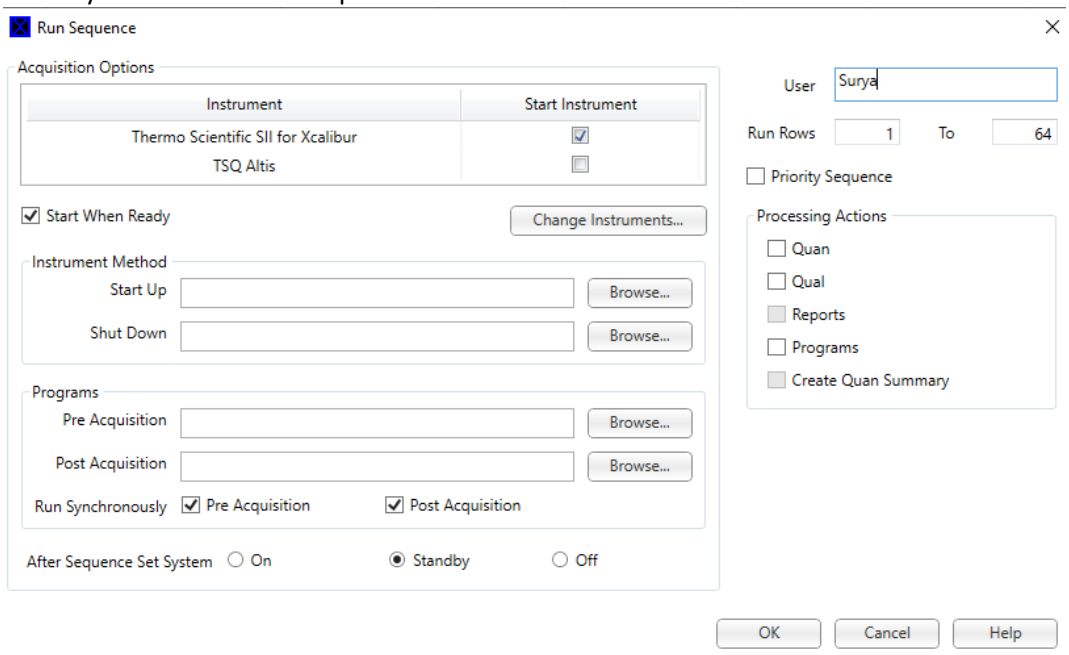
– Building a sequence

- To build a sequence (.sld file), open Xcalibur and click on Sequence Setup.
- Create a new sequence or start with an old sequence and change necessary information.
- Fill in the following fields: Sample ID, Sample Name, Dilution Factor, Position, Sample Type, Level, Laboratory (placeholder for calibrator and bench QC lot names), File Name, Path, Injection Volume, Instrument Method, and Comment.
- Check carefully for sample IDs and well positions.
- Sample ID and Sample Name columns generally should match.
- Check to make sure that appropriate instrument method is used. The injection volume is 50 µL. Dilution factor is adjusted as needed. Then, save the sequence file.

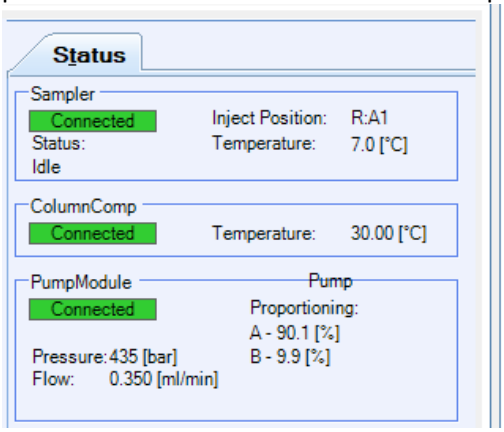
- In general, the layout of the sequence file is in the order of 4% albumin blank, 6-8 calibrators, one set of QC pools (low, medium and high), about 70 patient samples (for NHANES: 1 blind QC sample is inserted for every 20 patients), second set of QC set, and second set of calibrators.

– Running the sequence

- To run the sequence, open Xcalibur window. Click on “Run Sequence” button. In the pop-up window, change “User” to the generic instrument name. Change “Run Rows” to how many samples to run. Ensure these boxes are checked: “Thermo Scientific SII for Xcalibur - Start Instrument”, “Start When Ready”, “Run Synchronously: Pre-Acquisition and Post-Acquisition”. Most importantly, set “After Sequence Set System” to “Standby”. This will set the instrument to standby after the run is completed.



- Click on "Thermo Scientific SII for Xcalibur”. In “Status” tab, check the parameters and record the column back pressure.



- Click on “TSQ Altis”. In “Status” tab, check the parameters under “Vacuum” and record the Source Pressure and Analyzer Pressure. Refer to the instrument manual (see STARLIMS) for “TSQ Altis MS typical pressure readings”.
- From TSQ-Tune page, under “Detector” and record the “Dynode” (Gain should ideally be recorded while instrument is running a sample and the mass spec is scanning.)
- Use the “Acquisition Queue” tab to check how many samples are in queue and which sample is being injected. When all samples in the sequence have been injected, Acquisition Queue will be empty.
- The first injection (instrument check solution) is used to check for a good MS response (generally ion counts ~10^4) and a stable retention time.
- An albumin blank, which is extracted in the same manner as all patient, calibration, and QC samples, is injected during the run as an indicator of contamination.
- Background levels should be observed for the non-internal standard analytes, (generally ion counts ≤ 10^2).

(b) Maintenance

– Cleaning and Maintenance

All the following cleaning and maintenance tasks can be performed as needed.

- Check for air leaks in the MS system by listening for hissing sound and observing the parameters in the Tune window. Green square indicates a good readback value.
- Check Vacuum Pressure Levels. Refer to instrument manual (see STARLIMS) for source and analyzer pressure recommendations.
- Check the Argon and Nitrogen Gas supplies and replace the argon tank as needed. Refer to instrument manual (see STARLIMS) for argon and nitrogen reading recommendations.
- Check and replace any parts that are clogged (tubing, ion transfer tube, etc.). Refer to instrument manual (see STARLIMS) for relevant instructions.
- Check roughing pump oil by opening the pump ballast to allow the oil to drain back into the pump and close the ballast.
- Change the corona needle. Remove the old needle using tongs or tweezers and carefully handle the new needle with tongs and place it in the slot. Needle is sharp; thus, use caution and wear proper PPE.
- Clean the source housing to eliminate contaminants.
- Clean Ion Sweep Cone to the Spray Cone and change the ion transfer tube as needed
  - ◊ Turn off the flow of liquid from the LC (or other sample introduction device) to the API source.
  - ◊ Place the instrument in Standby mode.
  - ◊ Lower the ion transfer tube temperature and vaporizer temperature to 50 °C or less. Allow the ion transfer tube to cool.
  - ◊ Once the source reaches ambient temperature, remove the source housing from the front of the mass spectrometer.

- ◊ Carefully pull off the ion sweep cone after it is cooled enough to touch. Use methanol and a Kimwipe to wipe the inside and outside of the ion sweep cone. Thoroughly rinse with methanol. The cone may then be sonicated in methanol. Follow step-by-step instructions in the instrument's user manual (see STARLIMS) for cleaning ion sweep cone.
- ◊ Using appropriate tool, remove the ion transfer tube by turning it counterclockwise, then pull it out to free it from the ion source interface. Ion transfer tube can be replaced with a new one or cleaned by sonicating in methanol. Follow step-by-step instructions in the instrument manual (see STARLIMS) for cleaning ion transfer tube.
- ◊ Carefully remove all parts from the beakers after sonication, and blow-dry them with inert, oil-free gas.
- ◊ Wipe the spray cone with a lint-free tissue and methanol and inspect graphite o-ring for integrity. Replace if needed.
- ◊ Insert new or cleaned ion transfer tube into the heater block. Rotate while inserting and turn it clockwise until it is finger-tight.
- ◊ Reinstall ion sweep cone and source housing. Follow the instructions in the instrument's user manual (see STARLIMS).
  Note: Cleaning beyond the spray cone to Q00 requires care and is done infrequently. Refer to the TSQ Vantage Hardware manual (see STARLIMS) for instructions and diagrams.

(c) Preventative Maintenance (annually or semi-annually)

Preventative maintenance is performed by a service engineer and the following is included.

- Instrument calibration
- System performance checks by infusing the calibration solution
- Inspect and replace any parts if needed (pump seals, inlet filters, inlet valves, rotor seals, corona needle, etc.) and change pump oil

(d) Shutdown

See **JA-4027-I-04-Thermo LC-MS/MS Shutdown Instructions in Appendix B, section C.**

(e) Tuning and Calibration

Tuning and calibration can be performed as needed by user or a service engineer.

- Use Positive Ion Mode.
- Use Heated Electrospray Ionization (H-ESI source).
- Instrument calibration is performed annually or semi-annually as part of the preventive maintenance and is performed by the Service Engineer.
- Mass calibration checks can be conducted by user as needed using the calibration solution.
- The tuning and mass calibration of the first and second quadrupoles of the TSQ Altis using electrospray ionization (ESI) is performed by infusing a calibration

solution and running the instrument using Auto Tune-Calibration. Follow the instrument's user manual (see STARLIMS) for instructions on the calibration.

- The calibration solution is infused directly from the mass spectrometer using a Hamilton gas tight 500µL or 1000uL syringe at a flow rate of 5 to 10 µL/min.
- Peaks should be observed in Q1 and Q3 at mass 69.04, mass 102.13, mass 215.15, mass 622.03, mass 922.01 and mass 1521.97 in the positive ion polarity mode.
- The ion counts for the highest peak should be ~1x10^7.
- The source, voltage, etc. parameters can be adjusted to achieve maximal performance. Once the instrument is tuned, the compound dependent optimization may be conducted.

(f) Optimization (compound dependent)

Optimization can be performed as needed by user.

- Prepare a solution containing 50.0-125 nmol/L of each of the three analytes (25OHD3, epi-25OHD3, and 25OHD2) and each of the three internal standards (d6-25OHD3, d3-epi-25OHD3, and d3- 25OHD2) in 72% methanol: 28% water.
- Configure the Altis switching valve with a 5µL loop and syringe adapter as shown in the instrument's user manual (see STARLIMS).
- Load the 5µL loop with the solution with the switching valve in the 'load' position. To send the aliquot to the MS, switch the value to the 'inject' position. Do this for every condition that is being evaluated. Conduct 3 injections per condition to obtain an average signal. Investigate the temperatures, voltages, gas pressures, and the deck/probe position.
- Monitor each injection on the TIC plot to track the increase/decrease in signal per analyte that occurs with each conditional change. When maximal signal is achieved, set these conditions as the operating conditions.
- An infusion can be done with this solution to auto optimize "fast" parameters such as voltages and current. This cannot be done for the 'slow' change parameters such as temperatures and gas flows.

3) JA-4027-I-03-Hamilton Microlab STARlet Liquid Handler Operation for liquid-liquid extraction

(a) Serum samples:

(1) Check deck layout in current method file to see where all sample vial carriers, tip carriers, tube racks, solvent carriers, and well plate carriers are located.

(2) Reagent, tip and sample carrier positions on the deck are generally as shown below:

(a) For 8-channel system:

- ◊ Position 1-6: Tip_CAR_480_00 (Tip Carrier)
- ◊ Position 7-9: SMP_CAR_32_A00 (Sample Carrier for serum samples)
- ◊ Position 10-15: PLT_CAR_L5AC_00 (Plate Carrier)
- ◊ Position 16: RGT_CAR5x50_C (Reagent Carrier for internal standard & 72% methanol)
- ◊ Position 18-20: SMP_CAR_32_A00 (Sample Carrier for tall borosilicate tubes)
- ◊ Position 23: RGT_CAR_3R_A01 (Reagent Carrier for hexane)
- ◊ Position 25-30: TIP_CAR_480_00 (Tip Carrier)

(b) For 12-channel system:

- ◊ Position 1-4: Tip_CAR_288_C00 (Tip Carrier)
- ◊ Position 5-8: Tip_CAR_288_C00 (Tip Carrier)
- ◊ Position 14: RGT_CAR_3R_A01 (Reagent Carrier for hexane, internal standard & 72% methanol)
- ◊ Position 16-19: SMP_CAR_32_A00 (Sample Carrier for serum samples)
- ◊ Position 21-24: SMP_CAR_32_A00 (Sample Carrier for tall borosilicate tubes)
- ◊ Position 25-30: PLT_CAR_P3AC_01 (Plate Carrier)

(3) The 12-channel system has four sample carriers and four tall borosilicate tube carriers. Position #1-4 and 29-32 on these carriers are not used.

(4) Check the tip racks for 1000 µL tips and 300 µL tips on Tip Carriers at appropriate deck location on the worktable and restock if needed.

(5) Starting with position #1 for 8-channel system and #5 for 12-channel system, place labeled empty thin walled 13x100 borosilicate tubes (equal to the number of samples for analysis) onto Sample Carrier at the appropriate location on the worktable.

(6) Load all the solvents (Hexane, internal standard and 72% methanol/water) in the reagent carriers in their appropriate locations.

(7) Vortex and uncap all matrixed-based calibrators, unknowns, QCs, and blank samples ensuring that each cap is identified for the recapping process. Starting from position #1 (on 8-channel system) and #5 (on 12-channel system) on the sample carrier with inserts, load a blank, one set of calibrators and QCs, patient samples and a second set of QCs and calibrators.

(8) Go to the Microlab Star Run method icon on desktop, double click to open a workstation window.

(9) Go to [File] then open, locate the current method file→ double click on the file to open method→ Hit start {Green arrow icon})→Initialize will occur automatically → Prompt window appears How Many Samples? Enter the total number of vials → Edit tip count window appears → Look at Labware for tip size → Here, ensure 'First' and 'Last' standard volume tip counts are correct → OK → Prompt window appears → Look at Labware for tip size → Here, ensure 'First' and 'Last' high volume tip counts are correct → OK → Run will start at position 1 (for a routine run) maximum number of samples is 96 for a single well plate.

(10) The first few steps of the extraction mix the serum samples with internal standard, 72% methanol/water and hexane. Once the hexane is added a timer pops up and pauses the run. At this time, take all the tubes to a multi-tube vortexer and shake at speed 1,600 for 3 minutes with 1-minute rest in between for 3 shake cycles. Centrifuge the tubes for 5 minutes at 3,000 rpm.

(11) Return the tubes back to the Hamilton in their original positions.

(12) Continue the run by closing the timer.

(13) 1mL of hexane is transferred from each tube into a corresponding well of a 96 well plate. A 2nd timer pauses the run. Remove the well plate and dry down using TurboVap 96 [approximate Nitrogen Gas Time: 30 mins, Plate Temperature: typically 25°C, Flow Rate: 50-60 standard cubic feet per hours (SCFH)], Argonaut SPE Dry 96 (approximately Nitrogen Gas Time: 45 mins, Plate Temperature: ambient temperature, Flow Rate: 35 L/min AIR) or Speedvac to remove all the hexane.

(14) Return the dry plate to the liquid handler and place the plate in the original position.

(15) 300uL of 72% methanol/water is transferred to each dry well on the 96 well plate. At this point, the run on the liquid handler is completed.

(16) Remove all solvents, and store for subsequent runs. Remove tubes containing left-over samples and solvents from the Hamilton carriers and pour a few tubes at a time into an appropriate biohazard waste vessel. Discard empty tubes in biohazard pan. Remove and recap all samples and store appropriately.

(17) Open the Microlab StarLet through Method Editor as needed if method needs updates, changes or evaluations to check correct performance.

(b) Un-extracted solvent-based calibrators:

- This step can be performed on the same well plate with the serum but perform this last after the final serum reconstitution step is finished. Alternatively, prepare a second well plate solely for the calibrators.
- Follow step 1 as shown above for serum. Open the current calibrator method.
- Place 96-well plate in appropriate deck location.
- Thaw all calibrators. Then, vortex and load each vial in sample carrier. Starting from position #1 (on 8-channel system) on the sample carrier with inserts, load 2 sets of the calibrators.
- Load the internal standard in the reagent carrier, and place in appropriate deck location.
- Follow steps #8-9 as shown above for serum.
- After transfer of calibrators and internal standard, seal the plate. Take it to vortexer and gently shake content to mix for about 10 mins. Plate is ready for analysis.

(c) Extracted solvent-based calibrators:

(1) Follow step 1 as shown above for serum. Open calibrator method on each instrument.
(2) Starting with position #1 (on 8-channel system), place labeled empty thin walled 13x100 borosilicate tubes (equal to the number of calibrators for analysis) onto Sample Carrier at the appropriate location on the worktable.
(3) Thaw, then vortex all calibrators. Starting from position #1 on the sample carrier with inserts, load 2 sets of the calibrators.
(4) Follow steps #8-9 as shown above for serum.
(5) Transfer 100 uL solvent-based calibrators to 13x100 borosilicate tubes. These tube and vials will be either removed or repositioned before the extraction can proceed. See below for the steps.
    (a) Remove the 13x100 borosilicate tubes containing the calibrators from the current deck position and reposition them per the run sheet layout (i.e., 6-8 tubes in the front and 6-8 tubes in the back of the run).
    (b) Remove all the calibrator vials from the deck. Place either 4% albumin blank or DI-water vials equal to the number of calibrators in the front and in the back end of the run to match 5a positions.
    (c) Start the method to run the full sample set extraction (this includes all the QC, patients, blank, pre-aliquoted calibrators).
    (d) Immediately after the internal standard is added, pause the method and remove the 13x100 borosilicate tubes with calibrators from the deck. Cover them with parafilm and replace their spots on the desk with empty 13x100 borosilicate tubes to receive 72% MeOH/water, which we do not want to add to the calibrators. Then, un-pause to continue the extraction. (This step is done as a discard step so that all extraction tubes will contain the same volume since the calibration matrix is solvent-based (70% MeOH/ water).)
    (e) When this step (d) is over, discard the tubes that served as a place holder during the third step (d) in the extraction and replace them with the 13x100 borosilicate tubes containing calibrators back on the deck in their positions.
    (f) Allow the extraction to continue to the hexane step, which at that point, is the same as shown above in the serum extraction (see steps #10-17).

(d) Method-Specific Details:

- Label one 96-well plate with the date of the run. Also, prepare a run sheet and sequence showing each well position and associated sample IDs.
- Load samples, solvent reservoirs, borosilicate tubes, and well plate onto liquid handler deck in the proper order to match the run sheet, sequence and deck layout.
- Extraction Phase:
    (a) Use standard volume tips to pipette 100µL of blank, calibrators, QCs and unknown serum samples to 13x100 mm tubes. Standard volume tips are also used for transferring solvent-based calibrator to well plate.
    (b) Use standard volume tips to pipette 75µL of the IS solution to each tube/calibrator well and allow robotic mixing.
    (c) Use standard volume tips to pipette 100µL of 72% methanol to each serum tube and allow robotic mixing.

(d) Use high volume tips to pipette 1.5mL hexane to each tube (1mL hexane, discard tip, 500uL, discard tip). No robotic mixing is done at this step since the tubes will be manually shaken.

- Take the 13x100 mm tubes containing the above mixture to a multi-tube vortexer and shake the tubes at 1,600 shake speed for 3 minutes for 3 cycles. Allow 1-min rest between each cycle.
- Take the tubes to a centrifuge and spin for 5 minutes at 3,000 rpm to break up any emulsions that may have formed during shaking.
- Reconstitution Phase (for serum extraction only):
    - Return the tubes to the Hamilton in the correct order as designated in the sequence.
    - Use high volume tips to transfer 1mL of the hexane layer from each tube to the corresponding position in the 96-well plate.
- Take the well plate to either a Speedvac, Turbovap, or a plate dryer to evaporate off the hexane completely.
- Return the dried plate to the Hamilton for reconstitution and add 300 µL of 72% methanol to each dried cell.
- Take the reconstituted plate to a plate shaker and gently shake for 10 minutes.
- Cover the plate with a pre-slit silicone plate cover and load into the autosampler chamber set at 7°C.

4) JA-4027-I-04-Thermo LC-MS/MS Shutdown Instructions

The following is Thermo's emergency shutdown instructions for Thermo TSQ-Altis (Mass Spectrometer) and Vanquish System (Pump, Autosampler, and Column Compartment) during preventive maintenance or occasional power shutdown.

(a) Thermo TSQ-Altis LC-MS/MS:

- Ensure the instrument is not scanning, then turn off the PC.
- Before shutting the instrument down, open the ballast valves on the roughing pumps by turning the ballast valve (the black knob on right side of each pump) 90° to where the ridge is in line with the dots on the valve face ("open" position). Allow the pumps to ballast for ~10 minutes, then close the valve by turning it another 90° so that the ridge is not in line with the dots ("closed" position).
- After ensuring the valves are closed, switch off the power to the Electronics (the black switch) on the right side of the instrument. When the LEDs on the front panel are no longer lit, the Electronics are off.
- With the Electronics off, switch the Main Power breaker on the right side of the instrument to the "Off" position. The instrument will now vent.
- As an additional precaution, the main power cable may be unplugged from the right side of the instrument.
- NOTE: Nitrogen and Argon supplies do not need to be disconnected or turned off.
- To bring the instrument back under vacuum, repeat the previous steps in the reverse order (Plug in main power cord if applicable, switch Main Power 'On', switch Electronics 'On', Turn on the PC).
- Allow the system to pump down overnight (approximately 15 hours) before testing or resuming use.

(b) Vanquish Pump, Autosampler and Column Compartment:

- Prior to shutting the modules down, ensure all temperature-sensitive sample trays and well plates have been removed from the autosampler and that all pump flow is stopped.
- Turn off the PC.
- To power down the individual modules, the main power I/O switches for each module must be turned off.
- For autosamplers and pumps, these switches are located on the right side of the module at the rear.
- For column compartments, the switch is located on the rear of the module.
- As an additional precaution, the power cords may be unplugged from the right side of each module.
- Also, unplug the black power cord for the injector port.
- NOTE: Each module must be turned off to prevent potential issues.
- Switch the power switch to the "O" position.
- To power each module on, plug in the power cords (if applicable) and switch to the "I" positions.

5) JA-4027-I-05-Thermo LC-MS/MS Checking Mass Calibration on TSQ-Altis

(a) Preliminary Notes:

- To check mass calibration, the following supplies are needed:
  - ◊ calibration solution [Extended Mass Range (EMRS)]
  - ◊ 500-uL syringe
  - ◊ Tubing to connect syringe to probe
  - ◊ H-ESI probe
  - ◊ Syringe adapter
- Wear proper PPE.
- Do not wear nitrile gloves when working with the calibration solution! If contaminated, there will be a peak at 212 *m/z*.
- After using the calibration solution, discard the leftover solution from the syringe and rinse the syringe with acetonitrile.
- Refrigerate the EMRS container after opening. For long-term storage, keep it refrigerated (+2°C to +8°C).
- Refer to the instrument's operator manual for more detailed instructions. (see STARLIMS)

(b) Mass to check for in EMRS in the positive ion polarity mode:

- 69.04, 102.13, 215.15, 622.03, 922.01, 1521.97 m/z

(c) Setting up the probe:

- Turn off liquid flow to API source.
- Place mass spec in standby mode.
- Open Tune Window and click on the Ion Source pane.
- Set the Ion Transfer Tube Temperature and Vaporizer Temperature to 0°C.
- Check the readback temperatures. After the source cools to ambient temperature, remove the current APCI probe.
- Install the H-ESI probe.
- Adjust the probe positions to the following:
  - ◊ Depth: low to medium
  - ◊ Front-to-back position: closest to the MS entrance
  - ◊ Side-to-side position: center
- Switch the black knob for APCI needle to "off" position. This knob is located on the right side of the source housing.

(d) Setting up the Syringe and Determining initial API Source Settings:

- Fill a clean syringe (500 uL) with ERMS calibration solution, place it in the syringe pump, and connect it to the H-ESI probe.
- In the Tune window, place the mass spec in ON mode.
- Wait for the Ion Transfer Tube temperature to reach back to about 325°C.
- Click Profile (Centriod) and choose Profile data type.
- Determine the initial API Source settings:
  - ◊ In Tune Window, Click on Ion Source tab.
  - ◊ Set Current LC flow to 5 uL/min
  - ◊ Click Get Defaults

- ◊ Change Sheath Gas (Arb): 6
- ◊ Change Aux Gas (arb): 0 (OFF)
- ◊ Click Apply

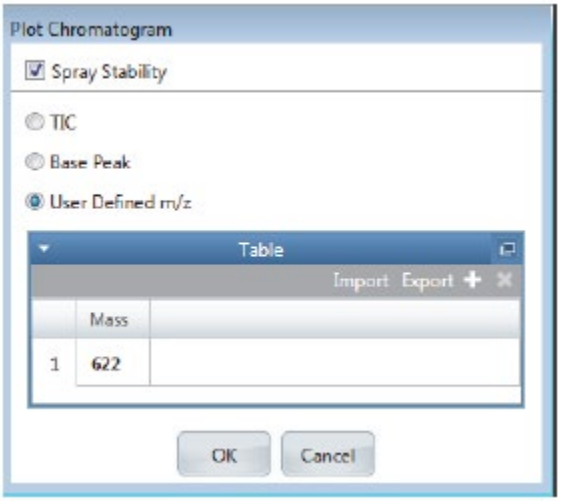
- Click **Syringe OFF** button to turn on the syringe pump. Only click once.
- Click the drop-down arrow button next to Syringe OFF button, and enter
  - ◊ Flow Rate (uL/min): 5
  - ◊ Volume (uL) : 500
  - ◊ click Apply.

- If no leaks are detected, press and hold Prime to prime the syringe (automatic setting: 100 uL/min). Verify that the system readback is normal.

(e) Evaluating the Spray Stability:

- Verify that current LC flow is 5 uL/min, ion polarity mode is positive, and data type is profile.
- In Tune window, Click on Define Scan pane.
- Type in the following settings:
  - ◊ Scan Type: SIM (Scan) Q1
  - ◊ Q1 Resolution (FWHM): 0.7
  - ◊ Scan Option: Scan Rate
  - ◊ Scan Rate: (Da/sec): 250
  - ◊ Scan Width (m/z): 10
  - ◊ CID Gas (mTorr): 0
  - ◊ Use Calibrated RF Lens: checked
  - ◊ Source Fragmentation: 30
- In Q1 SIM Scan table below, type in EMRS for compound and 622 for Precursor (m/z) to monitor 622 (ESI positive mode).

- Click on plot chromatogram button in the lower right of this window ( ).
- In Plot Chromatogram window, check Spray Stability box and check User Defined m/z and type 622 (positive mode) and click OK



- Observe the RSD of the target ion current. Review signal for stability. RSD <15% is acceptable. If signal stability rating is poor or RSD is above 15%, further optimize the API source parameters as follow to adjust stability:
  - ◊ adjust the sheath gas +/- 1 unit,
  - ◊ adjust spray voltage +/- 250 units,
  - ◊ change probe depth from L/M to M

(f) Checking Mass Calibration:

- Ensure the syringe pump is infusing the EMRS at 5uL/min to API source.
- Ensure the mass spec is in ON position.

- In Tune window, In Define Scan pane, set the following parameters:
  - ◊ Scan Type: Full Scan (Q1)
  - ◊ Scan Range (m/z): 50 to 1650
  - ◊ Q1 Resolution (FWHM): 0.7
  - ◊ Scan Rate (Da/sec): 1000
  - ◊ CID Gas (mTorr): 0
  - ◊ Source Fragmentation: 0
  - ◊ Click Apply

- In Tune window, In **Calibration** pane,
  - ◊ click Calibrate
  - ◊ choose Check Mass Calibration
  - ◊ Click Start
  - ◊ Look for the following m/z while it is running: 69.04, 102.13, 215.15, 622.03, 922.01, 1521.97 m/z
  - ◊ After calibration is done, click on Generate Report and click OK

- If this calibration fails, either contact the Service Engineer or proceed to:
  - ◊ Tune and Mass calibration
  - ◊ Once completed the calibration, proceed to EM gain calibration (To maintain sensitivity or restore it if it is lower.)

6) JA-4027-I-06-Hamilton Microlab STARlet Liquid Handler Calibration Verification using the Volume Field Verification (VFV) Kit

(a) Open method by double-clicking Microlab STAR Method Editor icon → [File] → Liquid Class Developer→ Select current method file.
(b) Look at the deck layout on the method to see where the tip carrier and weighing unit are located.
(c) Check the tip racks for 300 µL tips on Tip Carrier-Tip _CAR_480_A00, at appropriate deck location on worktable, and restock if needed (usually deck positions #16-21).
(d) Properly assemble the weighing unit and its components according to the manufacturer's instruction and plug in the corresponding cables from the weighing unit to the balance control, and from the balance control to the computer and power source.
(e) Place appropriate liquid you would like to test in the given vial and put it on the weighing unit.
(f) Go to the Microlab Star Run method icon on desktop. Double click to open a workstation window.
- Go to [File] then open, locate the current method file → double click on the file to open method → Hit start {Green arrow icon}) → Initialize will occur automatically → Prompt window appears Test Type Choice → Validation
- Which kind of tips would you like to test? Select the tip type → Edit tip count window appears → Look at Labware for tip size → Ensure that First and Last standard volume tip counts are correct → OK → Run will start at position 1
- Which liquid would you like to test? Select the liquid type
- Which liquid class would you like to test? Select the liquid class
- Liquid Transfer Information? Enter density volume (uL), liquid density (g/mL), total times transferred/channel
- Would you like to change the default pipetting settings? Select Yes
- Would you like to turn Anti-Droplet Control (ADC) on? Select No
- Would you like to re-use the tips? Select No
- What aspiration submerge depth would you like? Enter the appropriate submerge depth and click OK
- What dispense submerge depth would you like? Enter the appropriate submerge depth and click OK
- Aspiration mix volume, Number of mix Cycles on aspirate, Dispense mix volume, and Number of mix cycles on dispense → Enter appropriate values in each box and click OK

(g) Tips will be picked up automatically and weights of the set liquid volume will be measured with each tip while aspirating and dispensing the liquid from and into the original vial.
(h) Check the auto-generated data on the lab computer in the 'Liquid Class Results' file.
(i) The liquid class testing results are also backed up automatically to the local shared drive via ISLE.
(j) Calibration verification summary files are kept on the local shared drive.

D. Data Review

1) JA-4027-DR-01-Computerization & Data System Management

(a) Sample Identification
During sample preparation and analysis, samples are identified by sample IDs. A sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by sample collectors.

(b) Data Collection
Thermo UHPLC-MS/MS raw data files are collected and stored using instrument software (Xcalibur-Chromeleon or TraceFinder).

(c) Data Back-up
- Raw data files and sequence files from instrument computers are generally transferred via ISLE, KVM-switch or encrypted thumb drive to the local shared drive.
- Other instrument-related files from instrument computers (e.g., instrument method, processing method, etc.) are backed up automatically to the CDC network daily via an ISLE network. This is checked weekly by the analyst under the guidance of the project team leader.

(d) Peak Integration and Chromatographic Review
This assay uses two peak-integration methods:
- Ascent (Automated Integration Software) — Analysts upload sequence and raw data files to Ascent Online Portal, review auto-integrated peaks in Ascent as needed and retrieve a fully-processed excel result file, which is importable to STARLIMS, and a PDF file featuring chromatographic peaks for all analytes per run.

The technique for using Ascent automated integration software has been fully validated for use in method version 06. The validation data can be found on the local shared drive.

(e) Data Import
The final result files containing patient data as well as QC data are stored on the local shared drive. These files are further exported into STARLIMS Database for QC and statistical evaluation.

(f) STARLIMS Data Review
- Level I – Analyst
  - Double click the STARLIMS icon on desktop
  - Under ‘Run-based Tasks’, Select ‘Pending Runs Assigned to My Labs’
  - Choose ‘Show Pending Tests’ and select ‘4027 (Vitamin D, 25-hydroxy (Thermo 2))’
  - Click on ‘Add’ and select the Instrument
  - Run# and Equipment ID will be populated
  - [0] Run Instrument Macro – select the Ascent result file to run macro for STARLIMS import
  - [1] Upload Instrument File – import the post-macro result file to STARLIMS
  - [2] Mark Null Results – click this button to properly transfer null VID2 and VID3E results

- [3] Evaluate Sample QC – check the sample QC flags according to the defined criteria
- [4] Evaluate Run QC – evaluate bench QC via the DLS SAS Multi-Rule System QC program to determine QC pass/fail
- [5] Set Run QC Statuses – *set analytes pass/fail based on SAS out-of-control assessment*
- [6] Attach SAS QC file – upload both the SAS infile (.csv) and output file (.pdf)
- Enter run information in Run Comment and User Field 1-10
- Click on 'Manage Attachments' and upload Level I Checklist for the run
- Click 'Finish Results' located under the test workflow steps and notify General Supervisor (Team Lead)

– Level II – General Supervisor (Team Lead)
  - Double click the STARLIMS icon on desktop
  - Under 'Run-based Tasks', select 'Run Approval'
  - Choose 'Show Pending Tests' and select '4027 (Vitamin D, 25-hydroxy (Thermo 2))'
  - Review analyst run sheet, Level I Checklist and peak integration
  - Review sample QC evaluation
  - Review and confirm run QC evaluation
  - Assess and review blind QC results - click on 'Blind QC Results Only' tab, 'Assess Blind QC', choose 'Reported Value' for the blind QC assessment, select "Use Default Characterization Sets" and click 'Proceed to Next Step'
  - Print blind QC report - click on 'BQC Reports', 'All data displayed in the datagrid', 'A paper-based report from template', choose the current blind QC report format, click 'OK' and save the report as a PDF file and upload to STARLIMS
  - Enter bench QC (SAS) and blind QC evaluation status in the Run Comments column
  - Set results final - in 'All Results (S)' tab, click on 'Set Final' Wizard, select 'Process all samples displayed in the datagrid' and 'Run the Set Final Wizard' and click 'Proceed'
  - Choose set final criteria - check 'Required Sample QC Passed' and 'Required Run QC Passed'; check 'Pass' and 'Warn' for 'Allowable Results Statuses for Set Final'; choose date range to cover runs that include the previous analysis of these samples; click 'Proceed'
  - Resolve samples with retest results and set final
  - Submit sample IDs and repeat instructions to the analyst to schedlue the repeats
  - Click on 'Manage Attachments' and upload Level II Checklist for the run
  - In Run Approval tab, and click 'Release Run' and notify QA Officer

– Level III – QA Officer
  - Refer to Starlims Data Review Checklist Level III on the local shared drive

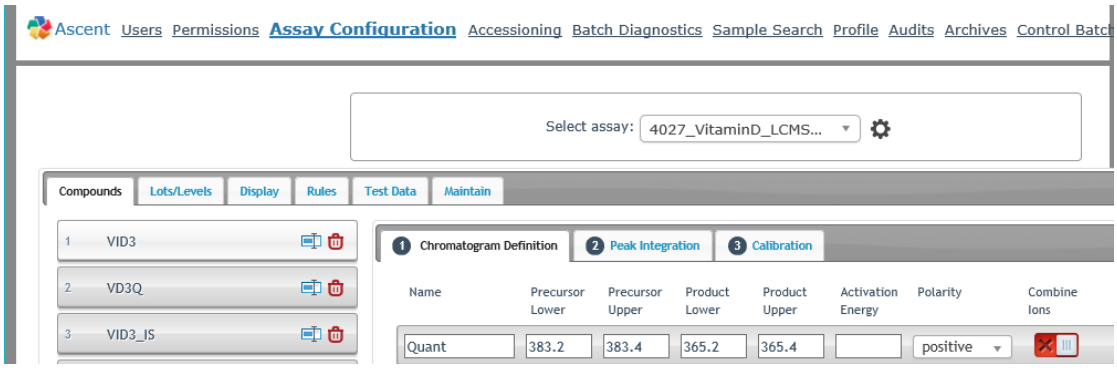
– General Supervisor (Lab Chief)
  - Conduct random "spot checks" to verify proper handling of lab results
  - Discuss with Team Lead or QA Officer course of action on difficult questions

2) JA-4027-DR-02-Ascent Data Review

Ascent is a software, used for automated integration and quantitation of raw data produced by the instrument.

A. How to configure an assay in Ascent for the first time

- Log into Ascent Portal. Click on **Assay Configuration** link to set up an assay.



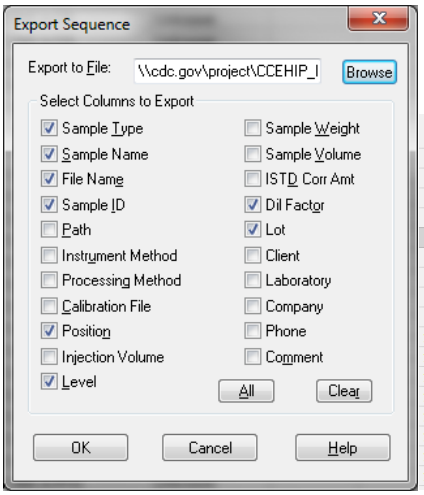
- In **Compounds** tab, create a list of compounds and set up peak integrating parameters for each compound.
- In **Chromatography Definition**, enter the mass and polarity of each compound.
- In **Peak Integration** tab, enter the retention time, peak thresholds, smoothing and other advanced settings per method requirements.
- In **Calibration** tab, enter degree, weighting, origin and response criteria.
- In **Lots/Levels** tab, enter a list of standards and QC lots and levels.
- In **Display** tab, define macros and instrument names.
- In Rules tab, select any pre-defined rules and set up the parameters for each rule.
- In **Test Data** tab, upload any file as a test run to test out the method.
- In **Maintain** tab, publish the method when all the configurations have been tested.

For Vitamin D method-specific configuration and settings, see the “production assay configuration” are saved on the network in the assay folder (#5-2-4).

B. How to Import raw files and sequence from an analytical run into Ascent

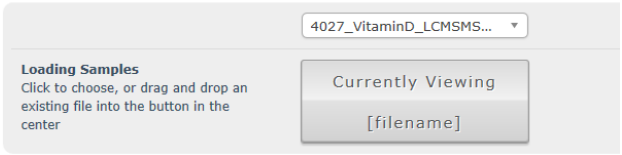
- Open an Xcalibur sequence file (.xld extension) and click on **File** and **Export Sequence.**
- When the Export Sequence window appears, check the following boxes: **Sample Type, Sample Name, File Name, Sample ID, Position, Level, Dilution Factor and Lot**. Then, click on Browse, and navigate to the location you wish to save the exported file (.csv), and name the file and click **OK**.

- The sequence will now be exported as a .csv extension file. Open that .csv file and make the following changes: delete the rows containing equilibration solution, change “L1 Lot” to “Lot” and make sure that the levels and the lot for calibrators and bench QCs are named the same as in Ascent software. Close this .csv file.



	1	2	3	4	5	6	7	8
1	Bracket Type=4							
2	Sample ID	Sample Name	Dil Factor	Position	Sample Type	Level	L1 Lot	File Name
3	Equil Solution 1	Equil Solution 1	1	B:A1	Unknown			08-09-2016_01
4	Equil Solution 2	Equil Solution 2	1	B:A1	Unknown			08-09-2016_02
5	VID-alb-blk	VID-alb-blk	1	A:A1	Blank			08-09-2016_03
6	VID-012016-cal1	VID-012016-cal1	1	A:B1	Std Bracket	cal1	VID-012016-cals	08-09-2016_04
7	VID-012016-cal2	VID-012016-cal2	1	A:C1	Std Bracket	cal2	VID-012016-cals	08-09-2016_05
8	VID-012016-cal3	VID-012016-cal3	1	A:D1	Std Bracket	cal3	VID-012016-cals	08-09-2016_06
9	VID-012016-cal4	VID-012016-cal4	1	A:E1	Std Bracket	cal4	VID-012016-cals	08-09-2016_07
10	VID-012016-cal5	VID-012016-cal5	1	A:F1	Std Bracket	cal5	VID-012016-cals	08-09-2016_08
11	VID-012016-cal6	VID-012016-cal6	1	A:G1	Std Bracket	cal6	VID-012016-cals	08-09-2016_09
12	VID-012016-cal7	VID-012016-cal7	1	A:H1	Std Bracket	cal7	VID-012016-cals	08-09-2016_10
13	LS12473b	LS12473b	1	A:A2	QC	Low	QC_2012b	08-09-2016_11
14	MS12474b	MS12474b	1	A:B2	QC	Medium	QC_2012b	08-09-2016_12
15	HS12475b	HS12475b	1	A:C2	QC	High	QC_2012b	08-09-2016_13
16	SRM 972a Lev1	SRM 972a Lev1	1	A:D2	Unknown			08-09-2016_14
17	SRM 972a Lev2	SRM 972a Lev2	1	A:E2	Unknown			08-09-2016_15

- Log into Ascent Portal Test Site using **Google Chrome** on CDC desktop or **Firefox** Web Browser through Citgo (https://cdctest.poweredbyascent.net/) using Ascent’s User ID and password.
- Click on **Accessioning** link located on top of the webpage. In the **Select the assay** dropdown menu, choose the assay based on the instrument used (for Surya: **4027_VitaminD_LCMSMS_Surya**)
- Click on **Currently Viewing [File Name]** button, and when **Choose File to Upload** window opens, select the .csv file that you created and click **open**.



- Ascent will display the uploaded sequence in Ascent format. **Choose the instrument (Surya)** from the dropdown menu and check to ensure that the samples are correctly recognized and press the **Ascent** button that is located at the bottom of the page.

- From the pop-up, save the Ascent-formatted sequence file (.csv) in the same location as the instrument raw data files for that run.
- Copy and paste the whole instrument file containing the raw files and the Ascent-formatted sequence file into the **TEST** folder in the Indigo Dropbox and wait until the file has been picked up automatically by Ascent portal.
- In Ascent portal, check if the run is imported by clicking on the **Ascent** logo button. Filter by your assay or instrument or other options to narrow down the runs.

C. How to review the analytical run in Ascent

- To review the imported run, click on the run link and click **review** button. Ascent automatically integrates the peaks, but analyst should review the automated integration of all peaks for all analytes, and manually integrate as needed.
- Calibration points can be excluded as needed using the calibration window. Check calibrator accuracy, Pass: % Difference from the target s ≤15%, ±30% (at the low end of the curve). Fail: If any of the high end of curve calibrators are >15% different to target, or multiple calibrators are flagged. Note: Excluding points for any given analyte on the curve is permitted due to spillage, instrument error, or random error as long as 5 points

remain on the curve. This will generally not be an issue since all front and back of run calibrators points (6-8 calibrator levels=12-16 points) are kept on the curve.

- Once the peak integration review is done, click on **Complete Review**.
- Analyst informs the assay leader that the run is ready for review and certification.
- Assay leader reviews the integration of all samples, the calibration curves and any points excluded from the curve. If multiple high calibration levels were excluded due to ≥15% difference from the target. Action: repeat the run.
- Assay leader reviews all calibration curves and confirms that they are acceptable (rules in section C above), documents all samples with potential interference and any integration issues for the analyst to correct.
- After the run is confirmed acceptable, assay leader **Certifies** it for STARLIMS import.
- Enter Ascent user ID and password, and the run review in Ascent is completed
- Analyst saves the CDC results.xlsx file and pdf file that Ascent produces in designated locations on the network and continue with STARLIMS import and data review.

3) JA-4027-DR-03-STARLIMS Data Review and Criteria

Staff Roles and Responsibilities in the STARLIMS Database:

Section	Category	Action	Staff Responsible	Spot Checking
1 1 2 2 3 3 3	Run-level	Step 0 – Run Macro Step 1 – Upload File Step 2 – Set Null Values Step 3 – Evaluate Sample QC Step 4 – Evaluate Run QC Step 5 – Set Run QC Statuses Sept 6 – Attach SAS QC File	Analyst	Lab Chief
4	Run-level	Detailed Review of Sample QC Review bench QC, and evaluate BQC (when available) Create a repeat report Set Final Wizard Release Run for Report	Project Lead	Lab Chief
5	Study-level	Set Reportable Report Results	QA Officer	Lab Chief

Details of responsibilities are depicted in the **JA-4027-DR-04-STARLIMS Flowchart.**

Testing of the STARLIMS importing and proper flagging is documented in the **Data Transfer Integrity File,** located in the branch network folder.

A. Prepare data for import into STARLIMS (Level I - Analyst)

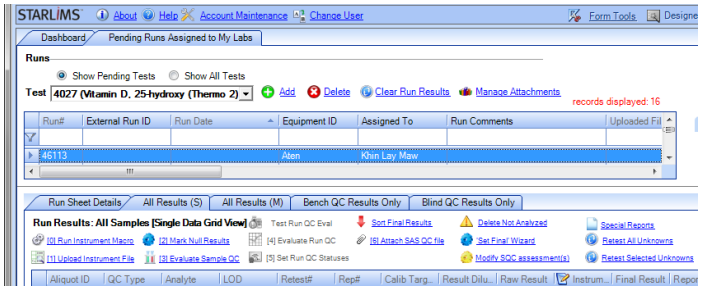
- Open Ascent result file to import into STARLIMS
- In **Calibration Data** tab, label column (K) as **cal 8?**, and if the run contains cal 8, enter “yes” for all 9 rows and if not, enter “no” for all 9 rows. This allows STARLIMS to recognize the highest calibrator and guide which calibrator criteria to use to evaluate the sample QC.
- In **Sample Data** tab, label column (AH) as **Sample Information**. Enter any sample-related information or internal comment codes in this column. For each sample, the same code or text must be entered for all analytes corresponding to that sample and their internal standards (9 rows). All codes and text comments entered in this column will show up in the **Sample Information** column in STARLIMS
- In **Sample Data** tab, label column (AI) as **NH Comment Code**. Enter NHANES comment codes such as 18, 21, 22, 23, 24, etc. in this column. For each sample, the same code must be entered for all analytes corresponding to that sample and their

internal standards (9 rows). All codes entered in this column will show up in the **NH Comment Code** column in STARLIMS

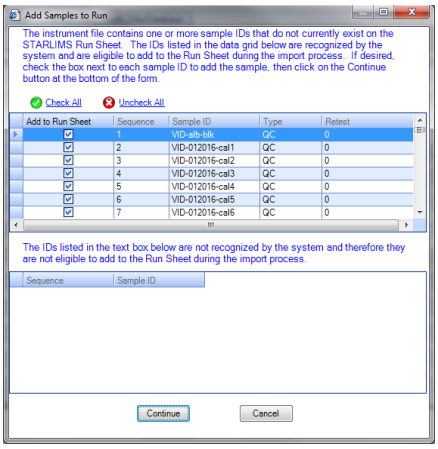
- Save this data file with a new name by removing "_CDC_results" from the file name

B. Data import into STARLIMS (Level I - Analyst)

- In **Run-based Tasks**, click on **Pending Runs Assigned to My Labs**
- Under **Test**, select: **4027 (Vitamin D, 25-hydroxy (Thermo 2))**
- To create a STARLIMS Run#, click **Add(+)**, select instrument (Surya) and click **OK**
- Select **All results (S)**



- Run instrument macro: **[0] Run Instrument Macro**
- In excel pop-up window, step 1: select instrument file, click **OK**
- Select the result file to convert, then click **OK**
- After the macro automatically runs, add **'Run#'** in the beginning of the converted file name and save the file under appropriate year in the assay specific folder in the instrument network "9_STARLIMS Import (after macro)".
- Close the post-macro file and proceed to upload it in STARLIMS
- Click on **[1] Upload Instrument File**
- Select post macro file to be imported, and click **Open**
- In the "Result Import" window, check that the number of samples being imported and the calibration data shown is correct and click **Upload**
- Under **Add Samples to Run** window, check if any samples are not recognized. If all samples are recognized, click Continue. Otherwise, click cancel and check the sample IDs in the import file and make corrections as needed and re-import the run. For sample IDs that are not in STARLIMS database, consult with the Data Manager to have them imported



- In **Results Not Imported** window, filter by **'compound'** and check that only **"_Q"** and **"_IS"** analytes are not imported and click Close
- Click **[2] Mark Null Results** to transfer properly null VID2 and VID3E results

C. Data review in STARLIMS (Level I - Analyst)

- Click **[3] Evaluate Sample QC** to flag each sample/analyte. This will flag each sample/analyte with **Pass, Warn, Check, and Fail**.
- Enter comment codes:
  - Sample specific: sample conditions and preparation issues (1-26 etc.)
  - Manual integration for calibrators, QC, SRM, CRM (764)
  - Excluded calibration points (1033)
- Check calibrator drift (>15% VID3 (S3-S8), VID2 (S4-S8), VID3E (S5-S8), >30% for the rest of the low end of the curve levels). Action: make a note.
- Check calibration curves (record slope, intercept and R^2). Verify that the slopes and intercepts are consistent with previous runs. R^2: Pass: >0.98, Check: >0.95 and ≤0.98 (requires branch chief approval), Fail: ≤0.95 (26): repeat the run.
- Check for contamination. Warn: blank >LOQ. Action: Notify TL.
- Verify analyte-specific sample QC flagging (listed below and summarized in the **Table** below).

Table. Method 4027: Analyte-specific Sample QC Criteria

Pass	Warn	Check	Fail	Incomplete
VID3: Result is ≥ 12.5 nmol and ≤ highest calibrator (nmol/L)	**VID3**: Result is < LOD nmol/L and needs repeat confirmation; add code 33 to indicate repeat confirmation	Confirmation ion ratio: **VID3**: ≤ 0.22 and ≥ 0.41 (for VID3 ≥ LOQ (LOQ = 1.32 nmol/L)). **VID2**: ≤ 0.34 and ≥ 0.63 (for VID2 ≥ LOQ (LOQ = 6.65 nmol/L)). **VID3E**: ≤ 0.24 and ≥ 0.44 (for VID3E ≥ LOQ (LOQ = 2.27 nmol/L)).	**VID3, VID2, VID3E**: Result is > highest calibrator (nmol/L), needs repeat with dilution; add code 32	**VID3, VID2, VID3E**: Data missing for proper flagging
VID2, VID3E: Result is ≥ LOD and ≤ highest calibrator (nmol/L)	**VID2, VID3E**: Result is < LOD (no need to repeat if no other failure suggests problems)	**VID3, VID2, VID3E**: ISTD % difference from mean for all unknowns is > 20%	**VID3, VID2, VID3E**: Instrument Result is < LOD on diluted sample (even if Final Result is ≥ LOD)	**VID3**: Result is < 12.5 nmol/L and needs repeat confirmation; add code 33 to indicate repeat confirmation
VID3, VID2, VID3E: Result is ≥ LOD and ≤ highest calibrator (nmol/L) and dilution > 1; add code 33 and 97 to indicate repeat and dilution confirmation if undiluted value >high cal		**VID3, VID2, VID3E:** Concentration ratio: ≤ 0.70 and ≥ 1.30 (for ≥ LOQ)	**VID3, VID2, VID3E:** Confirmation ion ratio and Concentration ratio out of range (for ≥ LOQ); add code 98	
VID3, VID2, VID3E: Instrument Result is ≥ LOD on diluted sample		**VID3, VID2, VID3E**: Relative retention time ≤ 0.98 and ≥ 1.02	**VID3, VID2, VID3E:** Result is null (check chromatography or repeat for confirmation)	
VID3, VID2, VID3E: Calibration curve R2 (>0.98)		**VID3, VID2, VID3E**: Calibration curve R^2 (>0.95 and ≤0.98) – requires Branch Chief's approval	**VID3, VID2, VID3E:** Calibration curve R^2 (≤0.95) – repeat run	

- Filter by **QC Type: N/A** (to separate out the unknown samples), then by **LOD** to evaluate sample-specific quality using sample QC criteria established during method validation. Document the number of flagged samples for each analyte in the following categories:
  - **ISTD area:** >20% difference from with-in run average. Check: review the integration and repeat the analysis of the sample.
  - **Null result (value=NF)**. Fail: code 26 and repeat.
  - **Relative retention time**, *RRT= Retention time of quantifier/ISTD:* 0.98-1.02 (applies to results ≥LOQ and ≤high cal). Check: review integration. Repeat if RRT out of bounds (OOB).
  - **Confirmation Ion Ratio**, *CIR = peak area of QI/peak area of CI*: 0.21 – 0.39 for VID3; 0.39 – 0.72 for VID2; and 0.21 – 0.38 for VID3E (applies to results ≥LOQ and ≤high cal). (***Limits:*** *within ±30% of the ion ratio mean calculated from thousands of sera from NHANES 2013-2014).* Check: review integration. Repeat if CIR out of bounds.
  - **Concentration Ratio** = *Concentration of QI/Concentration of CI*: 0.70-1.30 (*applies to results ≥LOQ and ≤high cal*). (***Limits:*** *within ±30% of 1.0 for all three analyte pairs*). Check: review integration. Repeat if CONC RATIO is out of bounds.
  - *Both CIR and CONC RATIO* out of bounds: Fail: repeat (98). If the result already has a code 98, report as **No reportable**, mark as **Null,** and notify QA officer.
  - **25(OH)D3 <12.5 nmol/L**. Incomplete: repeat (33). Warn: retested result, 'Repeated and confirmed', 33. Set Final (2^{nd} result) if 1^{st} vs. 2^{nd} result is within ±15%.
  - **25(OH)D3 <30 nmol/L**. Pass: code 0 (repeat to confirm is optional).
  - **Result >LOD and <lowest cal**. No flagging: report result
  - **Result <LOD**. Warn (37)
  - **Result >high cal**. Fail: repeat with dilution (32). Pass: repeated result with dilution >1 (97).
- Check internal and external reference material results for percent difference from the certified or reference values
- Enter sample-specific comment codes
- Select **[4] Evaluate Run QC.** DLS QC evaluation criteria *(Every run is evaluated via the DLS SAS Multi-Rule System QC program to determine QC pass/fail)*
  - Select **Use Default Characterization Sets**
  - Identify the date range to include at least 3 prior runs for the requisite minimum prior 10 results in the QC evaluation or keep it as a 3-month period as default in STARLIMS
  - Select **All results within this date range**
  - Select **All instruments**

- Click **Proceed to Next Step**
- In the QC Materials Results window, click **Start the SASQC Wizard**
- In the QC Evaluation Wizard window, click on **Save SAS Input File**
- Save the file in assay specific folder on the instrument network [organized by *Year → Quality Assurance Folder → Bench QC (SAS) Folder*].
- Name the file as "YYYY-MM-DD_Run#_SAS QC Report". Click **Save**, then **OK**
- In the QC Evaluation Wizard window, click on **Send to SAS Server**
- Save DLS QC Results PDF file in the same location and name as above
- Click **finished**

- Select **[5] Set Run QC Statuses** to set analytes pass/fail based on SAS out-of-control assessment
  - Select **Use Default Characterization Sets**
  - If all analytes passed, select **Set all results Pass.** If all analytes failed, select **Set all results Fail.** If some analytes passed and some failed, select **Set mixed result statuses** and choose the ones that passed in the table below. Click **Proceed.**
  - **Code 61** is added to any failed reportable analytes.
- Select **[6] Attach SAS QC file** and upload both the SAS infile (.csv) and output file (.pdf)
- Upload level 1 checklist and enter run-specific information into appropriate columns (Run Comment and User Field 1-10)
- Columns to enter run info:

Column	Run Information
Run Comment	SAS and Blind QC Status.
User Field 1	Calibrator Lot Used (e.g.: VID-102219-cal1-7)
User Field 2	NHANES group & position (e.g.: NH17-18 (N2524-070 #45-99) (N2525-070 #01-06) or NH17-18 (Repeats: N2501, N2518, N2519-070))
User Field 3	Corrective Action
User Field 4	Other studies/ samples included in run (e.g.: PT, SRM, CRM, etc.)
User Field 5	Liquid Handler Used for Extraction (e.g.: BVD, Apollo)
User Field 6	Internal Standard Lot Number (e.g.: IS_103020)
User Field 7	Column Lot, SN (e.g.: S18149, USEF001598)
User Field 8	Column Pressure (e.g.: 403.0 bar)
User Field 9	Flow Rate (e.g.: 350 uL/min)
User Field 10	Other method-related information

- Click **Finish Results** located under the test workflow steps and notify Project Lead

D. <u>Data Review (Level II – Project Lead)</u>

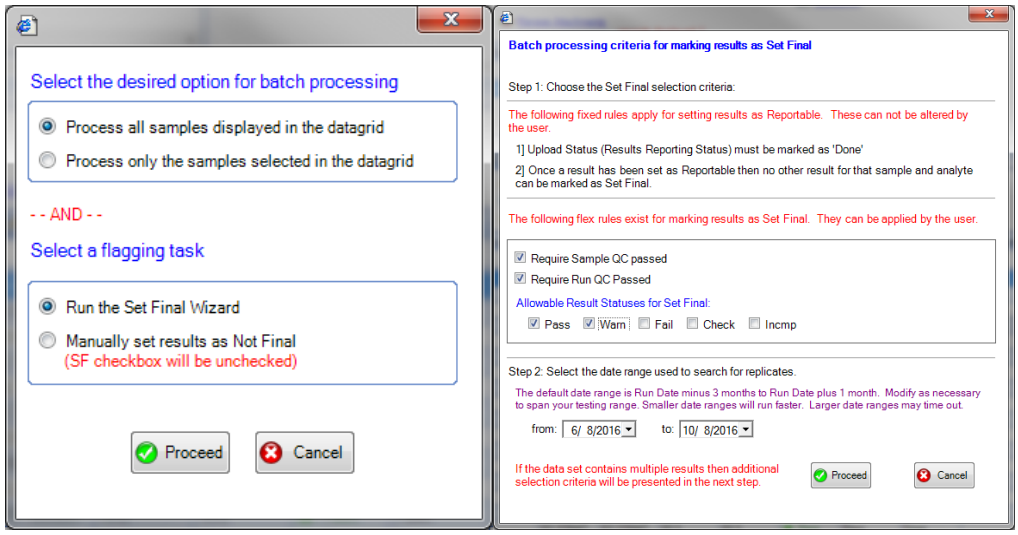
- Review Analyst's daily run sheet and checklist
- Review proper integration of at least 50% of QC and patient samples
- Review Sample QC Evaluation (Table above)
  - when >high cal, manually add code 32
  - when Pass and dilution >1, manually add code 97

- when there are two codes 33 and 97, set Final the result with code 97

- Review and confirm Run QC Evaluation (code 61, etc.)
- Assess and review the blind QC results.
  - Click on **Blind QC Results Only Tab**
  - Click on **Assess Blind QC**
  - Choose **Reported Value**
  - Select **Use Default Characterization Sets**
  - Click on **Proceed to Next Steps**
  - Click on **BQC Reports** to print the blind QC report
  - Save and upload the BQC report through **Manage Attachments** link

The use of blind QCs is optional but encouraged. Blind QCs are used in this method as a supplementary tool to assist in monitoring accuracy, precision, and aid in detecting errors such as sample misalignment; they are not used as part of the primary control procedures to determine if a run is out of control.

- Once review is finalized, enter the bench QC (SAS) and blind QC evaluation status for that run in the **Run Comments** column
- In **All Results (S)** tab, click on **'Set Final' Wizard** to set the results final
  - In the pop-up window, select **Process all samples displayed in the datagrid** and **Run the Set Final Wizard,** and click **Proceed.** In the next pop-up window, check **Required Sample QC Passed** and **Required Run QC Passed.** For the Allowable Results Statuses for Set Final, select **Pass** and **Warn**. Select the date range to cover runs that include the previous analysis of these samples. Click **Proceed.**



  - STARLIMS Alert window will inform of all results with only singlicate measures that were set final and the number of results with replicates that need to be resolved.
  - Samples with retest results will be displayed in a new window and can be resolved and set final from this window.

- ◊ The agreement between retest results should be typically within 85% for VID3 concentrations ≥ LOD and usually within 80% for VID2 and VID3E concentrations ≥ 3 times the LOD (VID2 LOQ= 6.65 nmol/L and VID3E LOQ= 2.27 nmol/L). In instances where a sample has low VID2 or VID3E levels near the low end of the calibration curve that exceed the 80% limit, supervisory approval may be obtained to set these samples 'final' and 'ready to report.' Otherwise, submit for repeat any sample that does not have agreement between multiple results → **Action:** Repeat - multi-analysis out-of-agreement
- ◊ Result that are set final may be coded based upon the reason to repeat (VID3 <12.5nmol/L = Code 33; repeated with dilution = Code 97, etc.)
- ◊ If multiple replicates are requested by studies, all replicates should be set final.

- Any result that does not have passing QC or is not reproducible in subsequent runs or continues to have persistent problems such as poor IS recovery result should be set as "No Reportable Result" with code 98 or 26 entered in the DLS comment code column.
- Submit sample IDs and repeat instructions to the analyst to schedlue the repeats.
- Click on **Manage Attachments** and upload Level II Checklist for the run
- In **Run Approval** tab, and click **Release Run**

E. Data Review (Level III – QA Officer)

Refer to Starlims Data Review Checklist Level III.

F. Data Review (General Supervisor – Lab Chief)

Conduct random "spot checks" to verify proper handling of lab results

Discuss with Team Lead or QA Officer course of action on difficult question

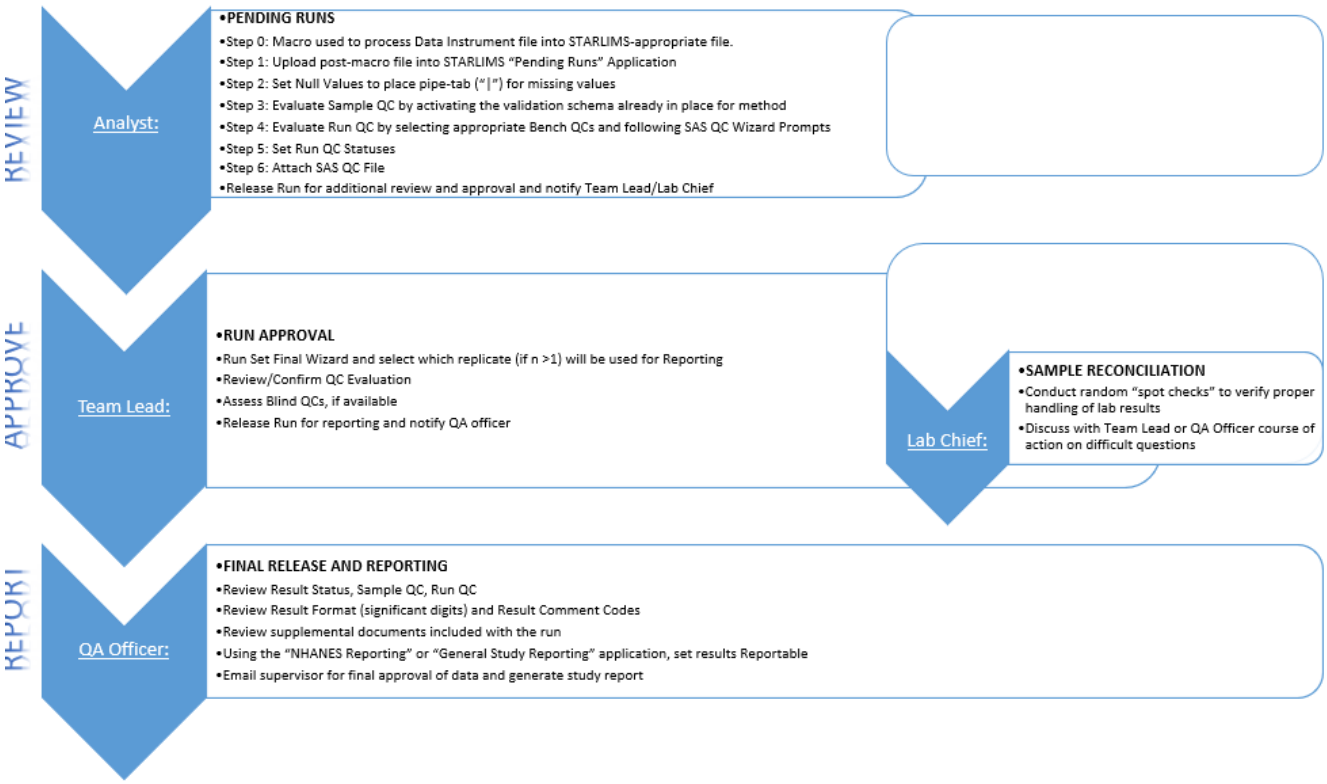
Data File Organization:

To organize the data files, same sets of folders are created each year in this assay specific folder on the instrument network.

	Folder Name	Contents in Folder
0	Quality Assurance	SAS Reports, Blind QC Reports and Data Review Checklists Level I & II
1	Run sheets	Analyst daily run sheets (contains extraction and analysis info)
2	Raw Data Files(.raw)	Raw output files from LC-MS/MS and Ascent sequence (.csv)
3	Xcalibur Sequences (.sld)	Xcalibur sequence (.sld) from LC-MS/MS
4	Xcalibur Sequences (.csv)	Xcalibur sequence converted from (.sld) to Xcalibur sequence (.csv)
5	Ascent Sequences (.csv)	Xcalibur sequence (.csv) converted to Ascent sequence (.csv)
6	Ascent Chromatography (.pdf)	Ascent Chromatography Output File
7	Ascent Results (.xls)	Ascent Result Output Files

8	STARLIMS Import (before macro)	Ascent Result Output Files with some info added (add cal 8 column, add NHANES comment codes, etc.)
9	STARLIMS Import (after macro)	Ascent Result Output Files ready for STARLIMS import

4) JA-4027-DR-04-STARLIMS Data Review Flowchart



5) JA-4027-DR-05-Out-of-control Corrective Action

(a) Bench QC (SAS) and blind QC status are noted in STARLIMS 'Run Comments' column with a list of analytes

(b) Investigating QC failures:
   a. Corrective Action Routine for SAS QC Failed Runs
   i. Verify there are no analyst remarks stating a problem in sample preparation of the QC in question.
      1. If there is a sample preparation problem, apply appropriate code and set sample QC rejected for QC in question and re-run SAS not including the sample QC rejected QC pool values. Else go on to step ii.
   ii. Verify integration of the QC pool for the failed analyte(s). If there is an integration problem, then fix the integration (and verify the same integration problem is not seen in other QC pools, the calibrators or unknowns; if so, then correct problem), re-create the excel file, re-import into database and re-run SAS. If there is not an integration problem, then go to step iii.
   iii. Verify internal standard recovery for the failed analyte(s) in the QC pools. To do this, look at the excel file and compare IS of QC pool with the run mean. If there is an IS recovery problem, apply code 1023 to the QC pool in question. On a case-by-case basis, evaluate whether it would be necessary to set sample QC rejected; therefore, eliminating the QC values when SAS is re-run, and also, evaluate the IS recovery in the patient samples and calibrators. Else go to step iv.
   iv. Verify calibration of the failed analyte(s)
      1. Verify calibration curve has an R^2 >0.98; if there is a calibration curve problem, verify integration for all calibration levels. If there is an integration problem, fix integration and re-create the excel file, re-import into database, and re-run SAS. If there is no integration problem, and R^2 is >0.95- ≤0.98, the run requires the Branch Chief's approval to pass. If R^2 is ≤0.95, the run fails.
      2. Compare the slope and intercept with the previous 3 runs on the same instrument and/or historical information on regression parameters. If the current failed run shows a slope or intercept difference, investigate why (i.e. response problem, IS issue, etc.) apply appropriate code if necessary.
   v. If the QC failure is true, then repeat the run for all analytes (VID3, VID2 and VID3E).

(c) QC failure corrective action write-up: User Field 3
QC failure corrective action: applied corrective action routine, "outcome."

Appendix C: Information on absorption maxima, absorption coefficient, and formulas to check the vitamin D concentration

Compound	Diluent for primary stock solution I	UV Absorption (Ref. 1 and 2*)		Molecular weight (g/mol)
		Absorption maximum (nm)	Molar extinction coefficient (L/mol*cm)	
25(OH)D3*	EtOH	265	18,300	400.7
25(OH)D2*	EtOH	265	19,400	412.7
3-epi-25(OH)D3	EtOH	265	18,300	400.7

(1) Primary stock solution is prepared as: dissolve ~1 mg in 10 mL ethanol (~0.250 mM) for 25(OH)D3 and 3 epi and ~1 mg in 50 mL of ethanol (~0.05 mM) for 25(OH)D2.

(2) Dilute the primary stock with EtOH (1:1) for 25(OH)D3 and 3Epi (dilution 1) and 2 times for 25(OH)D2 (dilution 2).

(3) Using a calibrated UV/vis spectrophotometer, measure the absorbance (AU) of each stock solution using ethanol as a reference blank.

(4) Calculate the actual concentrations of $25OHD_3$ and $25OHD_2$ in each stock solution by applying the following equation: **$C\ (ug/mL) = (AU * 1000 * Dilution * l * MW) / \varepsilon_{max}$**

   (a) AU is defined as absorbance units

   (b) c is the concentration

   (c) ε_{max} is the molar extinction coefficient

   (d) l is the path length, which in this case is 1 cm

   (e) MW is the molecular weight of the analyte

(5) To convert the concentration to SI units (nmol/L): multiply the mass concentration (ng/mL) by the following conversion factors: 2.4959 for 25(OH)D3 and 3Epi and 2.4233 for 25(OH)D2.

References for Appendix C:

1. Singh RJ:25-OH-Vitamin D in Methods in Clinical Chemistry. Kaplan and Pasce's: Clinical Chemistry: Theory, Analysis, Correlation (Hickman P. and Koerbi G), 2009 Australia.

2. Lensmeyer GL, Wiebe DA, Binkley N., Dresner MK. HPLC method for 25-hydroxyvitamin D measurement: comparison with contemporary assays, Clin Chem 2006; 52:1120-1126.

Appendix D: Transitions and LOD by Analyte in STARLIMS

m/z	Analyte Code	Analyte	Metric	Unit
395.30 > 377.30	VID2	25-hydroxyvitamin D2	Area	nmol/L
395.30 > 209.10	VD2Q	25-hydroxyvitamin D2 (Qualifier Ion)	Area	nmol/L
398.30 > 380.30	VID2_IS	d3-25-hydroxyvitamin D2	Area	
383.25 > 365.25	VID3	25-hydroxyvitamin D3	Area	nmol/L
383.25 > 105.00	VD3Q	25-hydroxyvitamin D3 (Qualifier Ion)	Area	nmol/L
389.25 > 371.25	VID3_IS	d6-25-hydroxyvitamin D3	Area	
383.25 > 365.25	VID3E	epimer-25-hydroxyvitamin D3	Area	nmol/L
383.25 > 105.00	VDEQ	epimer-25-hydroxyvitamin D3 (Qualifier Ion)	Area	nmol/L
386.30 > 368.30	VID3E_IS	d3-epi-25-hydroxyvitamin D3	Area	

See STARLIMS for current LODs.

Appendix E: 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. Currently, the CDC laboratory uses limits from LC-MS/MS TSQ Vantage on TSQ Altis. As needed, the limits will be recalculated and implemented to suit the new instrument specifications. Updated LOD and LOQ values from TSQ Altis are used as cut-off points for the rules.

References for Appendix E:

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.

Appendix F: 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	