NIST NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY
U.S. DEPARTMENT OF COMMERCE

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Standard Reference Material® 2973
Vitamin D Metabolites in Frozen Human Serum (High Level)
CERTIFICATE OF ANALYSIS

Purpose: This Standard Reference Material (SRM) is intended for use as an accuracy control in the critical evaluation of methods for determining the amount-of-substance concentration of vitamin D metabolites in human serum. This SRM can also be used as a quality assurance tool for assigning values to in-house control materials for these constituents.

Description: A unit of SRM 2973 consists of two vials of frozen serum at one concentration level of 25-hydroxyvitamin D_3 [25(OH)D_3] and 24R,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$]. Each vial of SRM 2973 contains approximately 1 mL of serum.

Certified Values: The certified values for 25(OH)D_3 and 24R,25$(OH)_2D_3$ are provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [1]. The certified values for 25(OH)D_3 and 24R,25$(OH)_2D_3$ are based on results from isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) procedures [2,3] performed at NIST. The NIST ID-LC-MS/MS methods are recognized as higher-order reference measurement procedures by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) [4]. The certified values are the method mean of the results from analyses at NIST via reference measurement procedures using ID-LC-MS/MS. The uncertainty provided with each certified value is an expanded uncertainty about the method mean that covers the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses, consistent with the Guide to the Expression of Uncertainty in Measurement [5]. The expanded uncertainties are calculated as $U = ku_c$, where u_c is the combined uncertainty and k is a coverage factor corresponding to approximately 95 % confidence for the analyte [5]. For the certified values shown in Table 1, $k = 2$. NIST certified values are traceable to the International System of Units (SI) derived unit of mass fraction, expressed as nanograms per gram; mass concentration, expressed as nanograms per milliliter; and amount of substance (molar) concentration, expressed as nanomoles per liter [1].

Table 1. Certified Values for 25(OH)D_3 and 24R,25$(OH)_2D_3$ in SRM 2973

Analyte	Mass Fraction (ng/g)	Mass Concentration (ng/mL)[a]	Amount Concentration (nmol/L)[b]
25-hydroxyvitamin D_3	38.6 ± 0.8	39.4 ± 0.8	98.4 ± 2.1
24R,25-dihydroxyvitamin D_3	3.06 ± 0.11	3.13 ± 0.11	7.51 ± 0.26

[a] The mass concentration level was calculated from the mass fraction using a measured serum density: 1.02229 ± 0.00002 g/mL (n = 3) at 23 °C.

[b] The molar concentration level was calculated from the mass concentration level using the relative molecular mass of 400.64 g/mol for 25(OH)D_3 and 416.64 g/mol for 24R,25$(OH)_2D_3$. The equivalent conversion factor is 2.4960 for 25(OH)D_3 and 2.4002 for 24R,25$(OH)_2D_3$.

Additional Information: Non-certified values and additional information are provided in Appendices A and B.

Period of Validity: The certified values delivered by **SRM 2973** are valid within the measurement uncertainty specified until **31 January 2028**. The certified values are nullified if the material is stored or used improperly, damaged, contaminated, or otherwise modified.

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Maintenance of Certified Values: NIST will monitor this SRM over the period of its validity. If substantive technical changes occur that affect the certification, NIST will issue an amended certificate through the NIST SRM website (https://www.nist.gov/srm) and notify registered users. SRM users can register online from a link available on the NIST SRM website or fill out the user registration form that is supplied with the SRM. Registration will facilitate notification. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

Safety: SRM 2973 IS INTENDED FOR RESEARCH USE. This is a human-source material. SRM 2973 is a Biosafety Level 2 material and should be handled according to applicable federal, state, and/or local regulations and according to policies and procedures of recipient's organization. The supplier has reported that each donor unit of serum used in the preparation of this product was tested by FDA-licensed tests and found to be negative for human immunodeficiency virus (HIV), HIV-1 antigen, hepatitis B, surface antigen, and hepatitis C [6].

This SRM was developed after an appropriate human subjects research determination by NIST.

Storage: Until required for use, SRM 2973 should be stored in the dark at a temperature between –20 °C and –80 °C.

Use: SRM 2973 is provided as a set of two vials of frozen serum. The vial (or vials) to be used should be allowed to thaw at room temperature for at least 30 min under subdued light. The contents of the vial should then be gently mixed prior to removal of a test portion for analysis. Precautions should be taken to avoid exposure to strong UV light and direct sunlight.

Other Information: Measurement of total 25(OH)D concentration in serum, the sum of 25-hydroxyvitamin D_2 [25(OH)D_2] and 25-hydroxyvitamin D_3 [25(OH)D_3], is generally considered a reliable indicator of vitamin D status. The concentration of 3-epi-25-hydroxyvitamin D_3 [3-epi-25(OH)D_3] is generally not included in total 25(OH)D, but this metabolite poses a potential measurement interference for some vitamin D metabolite assays. Measurement of 24R,25$(OH)_2D_3$ concentration in serum is considered as a catabolism marker and an indicator of kidney disease. For the majority of the U.S. population, serum concentrations for 25(OH)D typically range from 40 nmol/L to 75 nmol/L [7]. About 10 % of the population have 25(OH)D concentrations from 75 nmol/L to 120 nmol/L [7]. SRM 2973 was prepared specifically to provide a serum material with a 25(OH)D concentration near 100 nmol/L, which will complement the lower levels available in other SRMs with values assigned for 25(OH)D.

Commutability: SRM 2973 was distributed as a blinded study material in the Summer 2014 comparability study of the NIST/NIH Vitamin D Metabolites Quality Assurance Program (VitDQAP) Participants used both immunoassay (IA) techniques (chemiluminescence IA, enzyme IA, and radioimmunoassay) and liquid chromatographic (LC) techniques (LC with tandem mass spectrometry and LC with ultraviolet absorbance detection) to determine the 25(OH)D in SRM 2973. IA methods do not distinguish between the 25(OH)D_2 and 25(OH)D_3 metabolites, and the IA participants only reported values for total 25(OH)D [the sum of 25(OH)D_2 and 25(OH)D_3] in SRM 2973. Given that the concentration of 25(OH)D_2 is extremely low (Table A1) and below the limit of quantitation for most LC methods, the majority of the LC participants reported the same values for 25(OH)D_3 and total 25(OH)D. For the 63 values reported for total 25-hydroxyvitamin D using all methods (IA and LC), the median concentration value was 40.8 ng/mL with a percent coefficient of variation of 10 %. This median value agrees well with the NIST non-certified value of 40.1 ng/mL ± 0.8 ng/mL (Table A2) for total 25(OH)D. SRM 2973 is suitable for use with the majority of the methods used by VitDQAP participants.

In addition, SRM 2973 was distributed as a test material along with other SRMs and Proficiency Test/External Quality Assessment materials as part of the Vitamin D Standardization Program Commutability Study 2. Testing materials and 50 single-donor patient samples were characterized for 25(OH)D_2 and 25(OH)D_3 by a Reference Measurement Procedure at NIST [2]. Commutability of SRM 2973 for total 25(OH)D was assessed among the RMP values and values obtained from 28 institutions/laboratories using 20 ligand binding assay methods and 14 LC-MS/MS methods. The results indicated that SRM 2973 is commutable with all evaluated methods [8].

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Certificate Revision History: 25 March 2024 (Addition of non-certified value for $1\alpha,25(OH)_2D_3$; addition of commutability study information; editorial changes); 26 September 2023 (Editorial changes); 18 April 2023 (Change of period of validity; updated format; editorial changes); 14 December 2017 (Editorial changes); 11 September 2017 (Change from reference to certified values for $24R,25(OH)_2D_3$; editorial changes); 04 February 2016 (Original certificate issue date).

Certain commercial equipment, instruments, or materials may be identified in this Certificate of Analysis to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the Office of Reference Materials 100 Bureau Drive, Stop 2300, Gaithersburg, MD 20899-2300; telephone (301) 975-2200; e-mail srminfo@nist.gov; or the Internet at https://www.nist.gov/srm.

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APPENDIX A

Non-Certified Values: Non-certified values for 25(OH)D_2, 3-epi-25(OH)D_3, and 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] are provided in Table A1. Non-certified values are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods [1]. The non-certified values for 25(OH)D_2, 3-epi-25(OH)D_3, and 1α,25$(OH)_2D_3$ are based on the results from ID-LC-MS/MS procedures performed at NIST. The non-certified value for total 25(OH)D is provided in Table A2 primarily for assays that are suitable for use with SRM 2973 but do not measure the vitamin D metabolites separately (see section 'Commutability' for additional information).

Non-Certified Values for 25(OH)D_2, 3-epi-25(OH)D_3, and 1α,25-dihydroxyvitamin D_3: 25(OH)D_2 and 3-epi-25(OH)D_3 values are the method means of the results from analyses at NIST using ID-LC-MS/MS. The uncertainty provided with each non-certified value is an expanded uncertainty about the method mean to cover the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses, consistent with the Guide to the Expression of Uncertainty in Measurement [5]. The 1α,25$(OH)_2D_3$ value is the consensus value from three multiple reaction monitoring (MRM) approaches, which was determined using the Bayesian hierarchical Gaussian model of the NIST Consensus Builder [9]. The expanded uncertainties are calculated as $U = ku_c$, where u_c is the combined uncertainty and k is a coverage factor corresponding to approximately 95 % confidence for the analyte [5]. For the non-certified values shown in Table A1, $k = 2$.

Table A1. Non-certified Values for 25(OH)D_2, 3-Epi-25(OH)D_3, and 1α,25$(OH)_2D_3$ in SRM 2973

Analyte	Mass Fraction (ng/g)	Mass Concentration (ng/mL)[(a)]	Amount Concentration (nmol/L)[(b)]
25-hydroxyvitamin D_2	0.64 ± 0.02	0.65 ± 0.02	1.59 ± 0.05
3-epi-25-hydroxyvitamin D_3	2.05 ± 0.08	2.10 ± 0.08	5.23 ± 0.20
1α,25-dihydroxyvitamin D_3	0.055 ± 0.007	0.057 ± 0.007	0.137 ± 0.016

[(a)] Mass concentration levels were calculated from mass fractions using a measured serum density: 1.02229 g/mL ± 0.00002 g/mL (n = 3) at 23 °C.

[(b)] Molar concentration levels were calculated from mass concentration levels using the relative molecular masses. The relative molecular masses are 412.65 g/mol for 25(OH)D_2, 400.64 g/mol for 3-epi-25(OH)D_3, and 416.64 g/mol for 1α,25$(OH)_2D_3$. The equivalent conversion factors are 2.4234 for 25(OH)D_2, 2.4960 for 3-epi-25(OH)D_3, and 2.4002 for 1α,25-dihydroxyvitamin D_3.

Non-Certified Value for Total 25(OH)D: Vitamin D levels in serum are typically reported as the total of 25(OH)D_3 and 25(OH)D_2. The value for total 25(OH)D as the sum of the individual values for 25(OH)D_3 and 25(OH)D_2 is shown in Table A2. The uncertainty provided with the value is an expanded uncertainty about total 25(OH)D to cover the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses and their respective uncertainties of the two analytes, consistent with the Guide to the Expression of Uncertainty in Measurement [5]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty and k is a coverage factor corresponding to approximately 95 % confidence. For the value shown in Table A2, $k = 2$.

Table A2. Non-certified Value for Total 25(OH)D in SRM 2973[(a)]

Analyte	Mass Fraction (ng/g)	Mass Concentration (ng/mL)[(b)]
Total 25(OH)D	39.2 ± 0.8	40.1 ± 0.8

[(a)] The value is denoted as a non-certified value based on the combination of a non-certified value for 25(OH)D_2 and a certified value for 25(OH)D_3.

[(b)] The mass concentration level was calculated from the mass fraction using a measured serum density: 1.02229 g/mL ± 0.00002 g/mL (n = 3) at 23 °C.

Period of Validity: These non-certified values are valid until **31 January 2028** within the measurement uncertainty specified. The value assignments are nullified if this material is stored or used improperly, damaged, contaminated, or otherwise modified.

Maintenance of Non-Certified Values: NIST will monitor this material to the end of its period of validity. If substantive technical changes occur that affect the non-certified values during this period, NIST will update this Appendix and notify registered users. SRM users can register online from a link available on the NIST SRM website or fill out the user registration form that is supplied with the SRM. Registration will facilitate notification. Before making use of any of the values delivered by this material, users should verify they have the most recent version of this documentation, available through the NIST SRM website (https://www.nist.gov/srm).

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APPENDIX B

Support for the development of SRM 2973 was provided in part by the National Institutes of Health Office of Dietary Supplements (NIH-ODS).

Source and Preparation: SRM 2973 was prepared by Solomon Park Research Laboratories (Kirkland, WA). One serum pool was prepared. The naturally occurring concentrations of vitamin D metabolites in the human serum pool used to prepare this SRM have not been modified.

Analysis: Value assignment of the concentrations of 25(OH)D_3, 25(OH)D_2, 3-epi-25(OH)D_3, 24R,25$(OH)_2D_3$, and 1α,25$(OH)_2D_3$ in SRM 2973 were based on the results from ID-LC-MS/MS measurements at NIST.

Measurement of 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 by ID-LC-MS/MS (NIST): Serum (1.0 g to 2.0 g) was spiked with an appropriate internal standard solution ($^2H_6$-25(OH)D_3, $^2H_3$-25(OH)D_2, or $^2H_3$-3-epi-25(OH)D_3). After equilibration at room temperature for 1 h, the pH of each sample was adjusted to pH 9.8 ± 0.2 with carbonate buffer. Analytes were extracted twice from the serum matrix with a mixture of hexane and ethyl acetate. The combined extracts were dried under nitrogen at 45 °C, and the residues were reconstituted with methanol for LC-MS/MS analysis. Extracts were analyzed using either an Ascentis Express F5 Pentafluorophenylpropyl (Supelco, Bellefonte, PA) or a Zorbax SB-CN cyanopropyl (Agilent Technologies, Palo Alto, CA) column under isocratic conditions with water:methanol mobile phases. Atmospheric pressure chemical ionization (APCI) in the positive-ion mode and multiple reaction monitoring (MRM) mode were used. The following transitions were monitored: *m/z* 401 → *m/z* 383 for 25(OH)D_3 and 3-epi-25(OH)D_3; *m/z* 407 → *m/z* 389 for $^2H_6$-25(OH)D_3 and $^2H_6$-3-epi-25(OH)D_3; *m/z* 413 → *m/z* 395 for 25(OH)D_2; and *m/z* 416 → *m/z* 398 for $^2H_3$-25(OH)D_2.

Measurement of 24R,25$(OH)_2D_3$ by ID-LC-MS/MS (NIST): Serum (1.5 g to 2.0 g) was spiked with an internal standard solution containing $^2H_6$-24R,25(OH)D_3. After equilibration at room temperature for 1 h, the pH of each sample was adjusted to pH 9.8 ± 0.2 with carbonate buffer. The 24R,25(OH)D_3 was extracted twice from the serum matrix with a mixture of hexane and ethyl acetate. The combined extracts were dried under nitrogen at 45 °C, and the residues were reconstituted with methanol for LC-MS/MS analysis. Extracts were analyzed using an Ascentis Express C_{18} column under isocratic conditions with a water:methanol mobile phase. APCI in the positive-ion mode and multiple reaction monitoring (MRM) mode were used. The following transitions were monitored: *m/z* 417 → *m/z* 381 for 24R,25$(OH)_2D_3$ and *m/z* 423 → *m/z* 387 for $^2H_6$-24R,25$(OH)_2D_3$.

Measurement of 1α,25$(OH)_2D_3$ by ID-LC-MS/MS (NIST): Serum (1.0 g to 2.0 g) was spiked with an internal standard solution containing $^{13}C_3$-1α,25$(OH)_2D_3$. After equilibration at room temperature for 1 h, the pH of each sample was adjusted with carbonate buffer. The 1α,25-dihydroxyvitamin D_3 was extracted twice from the serum matrix with a mixture of hexane and ethyl acetate. The combined extracts were dried under nitrogen at 40 °C, and the residues were reconstituted with a mixture of methanol and water for LC-MS/MS analysis. Extracts were analyzed using an Ascentis Express C_{18} column under isocratic conditions with a water:methanol mobile phase containing lithium acetate as an additive. Electrospray ionization in the positive-ion mode and MRM mode were used. The following transitions were monitored: *m/z* 423.3 → *m/z* 369.0 and *m/z* 423.3 → *m/z* 387.3 for 1α,25$(OH)_2D_3$ and *m/z* 426.2 → *m/z* 372.0 and *m/z* 426.2 → *m/z* 390.3 for $^{13}C_3$-1α,25$(OH)_2D_3$.

Homogeneity Analysis: The homogeneity assessment was made at the time the certification analyses were performed for each of the individual analytes. A stratified sampling plan was devised to test for homogeneity across the entire lot of vials. There was no apparent trend in the data when plotted against the sequence in which the vials were filled.

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