National Institute of Standards and Technology
U.S. Department of Commerce

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Standard Reference Material® 972a
Vitamin D Metabolites in Frozen Human Serum
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 972a consists of four vials (Levels 1 through 4) of frozen serum with different concentration levels of 25-hydroxyvitamin D [25(OH)D] and 24R,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$]. Each vial of SRM 972a contains approximately 1 mL of serum.

Certified Values: The certified values for 25$(OH)D_3$, 25$(OH)D_2$, 3-epi-25$(OH)D_3$, and 24R,25$(OH)_2D_3$ are provided in Table 1, certified values for total 25(OH)D are provided in Table 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$(OH)D_2$, 25$(OH)D_3$, 3-epi-25$(OH)D_3$, and 24R,25$(OH)_2D_3$ in SRM 972a

	Mass Fraction (ng/g)	Mass Concentration (ng/mL)[a]	Molar Concentration (nmol/L)[b]
Level 1			
25-hydroxyvitamin D_3	28.1 ± 1.1	28.8 ± 1.1	71.8 ± 2.7
3-epi-25-hydroxyvitamin D_3	1.77 ± 0.10	1.81 ± 0.10	4.5 ± 0.2
24R,25-dihydroxyvitamin D_3	2.60 ± 0.10	2.66 ± 0.10	6.38 ± 0.23
Level 2			
25-hydroxyvitamin D_2	0.79 ± 0.08	0.81 ± 0.06	2.0 ± 0.2
25-hydroxyvitamin D_3	17.7 ± 0.4	18.1 ± 0.4	45.1 ± 1.0
3-epi-25-hydroxyvitamin D_3	1.25 ± 0.09	1.28 ± 0.09	3.2 ± 0.2
24R,25-dihydroxyvitamin D_3	1.38 ± 0.05	1.41 ± 0.05	3.39 ± 0.12
Level 3			
25-hydroxyvitamin D_2	12.9 ± 0.3	13.2 ± 0.3	32.0 ± 0.8
25-hydroxyvitamin D_3	19.4 ± 0.4	19.8 ± 0.4	49.5 ± 1.1
24R,25-dihydroxyvitamin D_3	1.58 ± 0.06	1.62 ± 0.06	3.88 ± 0.13
Level 4			
25-hydroxyvitamin D_3	28.8 ± 0.9	29.4 ± 0.9	73.4 ± 2.3
3-epi-25-hydroxyvitamin D_3	25.4 ± 2.1	26.0 ± 2.2	64.8 ± 5.4
24R,25-dihydroxyvitamin D_3	2.58 ± 0.09	2.64 ± 0.09	6.32 ± 0.22

[a] Mass concentration levels were calculated from mass fractions using measured serum densities: Level 1, 1.02326g/mL ± 0.00002g/mL; Level 2, 1.02196g/mL ± 0.00002g/mL; Level 3, 1.02294g/mL ± 0.00001g/mL; and Level 4, 1.02295g/mL ± 0.00011g/mL.

[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 25$(OH)D_3$ and 3-epi-25$(OH)D_3$ and 416.64 g/mol for 24R,25$(OH)_2D_3$. The equivalent conversion factors are 2.4234 for 25$(OH)D_2$, 2.4960 for 25$(OH)D_3$ and 3-epi-25$(OH)D_3$ and 2.4002 for 24R,25$(OH)_2D_3$.

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Table 2. Certified Values for Total 25(OH)D in SRM 972a[(a)]

	Mass Fraction (ng/g)	Mass Concentration (ng/mL)[(b)]
Level 2		
Total 25(OH)D	18.5 ± 0.4	18.9 ± 0.4
Level 3		
Total 25(OH)D	32.3 ± 0.5	33.0 ± 0.5

[(a)] Certified values for total 25(OH)D are based on the combination of certified values for both $25(OH)D_2$ and $25(OH)D_3$.
[(b)] Mass concentration levels were calculated from mass fractions using measured serum densities: Level 2, 1.02196g/mL ± 0.00002g/mL ; and Level 3, 1.02294g/mL ± 0.00001g/mL.

Non-Certified Values: Non-certified values for SRM 972a are provided in Appendix A.

Additional Information: Additional information is provided in Appendix B.

Period of Validity: The certified values delivered by **SRM 972a** 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.

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 of Analysis 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 972a IS INTENDED FOR LABORATORY RESEARCH USE ONLY. THIS IS A HUMAN SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier of the serum has reported that each donor unit of serum used in the preparation of this product has been tested by a FDA-approved method and found non-reactive/negative for hepatitis B surface antigen (HbsAg), human immunodeficiency (HIV) 1 and 2 antibodies, and hepatitis C virus (HCV). However, no known test method can offer complete assurance that hepatitis B virus, hepatitis C virus, HIV, or other infectious agents are absent from this material. Accordingly, this human blood-based product should be handled at the Biosafety Level 2 or higher as recommended for any POTENTIALLY INFECTIOUS HUMAN SERUM OR BLOOD SPECIMEN in the Centers for Disease Control and Prevention/National Institutes of Health Manual [2].

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

Storage: Until required for use, SRM 972a should be stored in the dark at a temperature between –20 °C and –80 °C.

Use: SRM 972a is provided as a set of four 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 $24R,25(OH)_2D_3$ in serum is considered as a catabolism marker and an indicator of kidney disease.

Each of the four levels of SRM 972a was prepared with a specific target level of 25(OH)D. While some measurement methods might be applicable to each of the four levels of SRM 972a, it is recognized that some methods may not be applicable to some levels. Individual users will need to assess which level or levels best suit their particular needs. Levels 1, 2, and 3 of SRM 972a were prepared from pools of human serum with endogenous concentrations of 25(OH)D. Level 4 was prepared from a pool of human serum that was fortified with 3-epi-25-hydroxyvitamin D_3.

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Certificate Revision History: 20 April 2023 (Change of period of validity; updated format; editorial changes); 31 March 2021 (Certified values for 25-hydroxyvitamin D_2 [$25(OH)D_2$] updated for Level 2 and Level 3 in Table 1; certified values for total 25-hydroxyvitamin D [25(OH)D] updated for Level 3 in Table 2; editorial changes); 29 November 2017 (Correction to tables numbering; editorial changes); 11 September 2017 (Change from reference to certified values for $24R,25(OH)_2D_3$; editorial changes); 15 September 2015 (Addition of reference values for $24R,25(OH)_2D_3$; addition of certified and reference values for total 25(OH)D; update of certified and reference values for $25(OH)D_3$ and 3-epi-$25(OH)D_3$; editorial changes); 20 February 2013 (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 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]. Non-certified values are provided in Tables A1 and A2.

Table A1. Non-Certified Values for 25(OH)D_2 and 3-epi-25(OH)D_3 in SRM 972a

	Mass Fraction (ng/g)	Mass Concentration (ng/mL)[a]	Molar Concentration (nmol/L)[b]
Level 1			
25-hydroxyvitamin D_2	0.52 ± 0.06	0.54 ± 0.06	1.3 ± 0.2
Level 3			
3-epi-25-hydroxyvitamin D_3	1.14 ± 0.14	1.17 ± 0.14	2.9 ± 0.4
Level 4			
25-hydroxyvitamin D_2	0.54 ± 0.10	0.55 ± 0.10	1.3 ± 0.2

[a] Mass concentration levels were calculated from mass fractions using measured serum densities: Level 1, 1.02326g/mL ± 0.00002g/mL; Level 3, 1.02294g/mL ± 0.00001g/mL; and Level 4, 1.02295g/mL ± 0.00011g/mL.

[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 and 400.64 g/mol for 25(OH)D_3 and 3-epi-25(OH)D_3. The equivalent conversion factors are 2.4234 for 25(OH)D_2 and 2.4960 for 25(OH)D_3 and 3-epi-25(OH)D_3.

Table A2. Non-Certified for Total 25(OH)D in SRM 972a[a]

	Mass Fraction (ng/g)	Mass Concentration (ng/mL)[b]
Level 1		
Total 25(OH)D	28.7 ± 1.1	29.3 ± 1.1
Level 4		
Total 25(OH)D	29.3 ± 0.9	30.0 ± 0.9

[a] Non-certified values for total 25(OH)D are based on the combination of a non-certified value for 25(OH)D_2 and a certified value for 25(OH)D_3.

[b] Mass concentration levels were calculated from mass fractions using measured serum densities: Level 1, 1.02326g/mL ± 0.00002g/mL; and Level 4, 1.02295g/mL ± 0.00011g/mL.

Period of Validity: The non-certified values are valid within the measurement uncertainty specified until **31 January 2028.** The value assignments are nullified if the 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 Certificate of Analysis 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 972a was provided in part by the National Institutes of Health Office of Dietary Supplements (NIH-ODS). Technical consultation was provided by C.T. Sempos, J.M. Betz and P.M. Coates of NIH-ODS.

Source and Preparation: SRM 972a was prepared by Solomon Park Research Laboratories (Kirkland, WA). Four serum pools were prepared. The naturally occurring concentrations of vitamin D metabolites in the human serum pools used to prepare Levels 1, 2, and 3 have not been modified. Level 4 is a human serum pool enriched with 3-epi-25(OH)D_3.

Analysis: Value assignment of the concentrations of 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 in SRM 972a are based on the combination of results provided from two analytical methods at NIST (ID-LC-MS and ID-LC-MS/MS) and from two ID-LC-MS/MS analytical procedures at CDC. Value assignment of the concentrations of 24R,25$(OH)_2D_3$ are based on the results from ID-LC-MS/MS at NIST.

Measurement of 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 by ID-LC-MS (NIST): Serum (450 mg) and an internal standard solution containing $^2H_6$-25(OH)D_3, $^2H_3$-25(OH)D_2, and $^2H_3$-3-epi-25(OH)D_3 were combined in glass tubes, proteins were precipitated, and the metabolites were extracted into hexane twice. The hexane phases were combined and evaporated to dryness at 40 °C under nitrogen. The residues were reconstituted with methanol and vortex-mixed. Extracts were analyzed by using LC-MS with (1) an Ascentis Express F5 pentafluorophenylpropyl column (Supelco, Bellefonte, PA) and (2) a Zorbax SB-CN cyanopropyl stationary phase column (Agilent Technologies, Palo Alto, CA). Analyses on the pentafluorophenylpropyl column were performed under isocratic conditions with a mobile phase of 26 % water and 74 % methanol at a flow rate of 0.8 mL/min. All solvent compositions represent volume fractions in percent. The column temperature was maintained at 15 °C. A step gradient to 100 % methanol was incorporated into the method at the end of the run to elute retained matrix components. Analyses on the cyanopropyl column were performed under isocratic conditions with a mobile phase of 33 % water and 67 % methanol (Levels 1, 2, and 4 of SRM 972a) or 34 % water and 66 % methanol (Level 3) at a flow rate of 1.0 mL/min. A step gradient to 100 % methanol was incorporated into the method at the end of the run to elute retained matrix constituents. The column temperature was maintained at 45 °C.

Atmospheric pressure chemical ionization (APCI) with positive polarity was used for both chromatographic methods. The $[M - H_2O + H]^+$ ions were monitored and used for quantification of all species. The ions monitored included *m/z* 383 for 25(OH)D_3 and for 3-epi-25(OH)D_3; *m/z* 386 for $^2H_3$-3-epi-25(OH)D_3; *m/z* 389 for $^2H_6$-25(OH)D_3; *m/z* 395 for 25(OH)D_2; and *m/z* 398 for $^2H_3$-25(OH)D_2.

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 or a Zorbax SB-CN column under isocratic conditions with water:methanol mobile phases. 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; *m/z* 404 → *m/z* 386 for $^2H_3$-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 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 by ID-LC-MS/MS (CDC): Samples of SRM 972a (100 µL) were spiked with the following isotopically-labeled internal standards: $^2H_6$-25(OH)D_3, $^2H_3$-25(OH)D_2, and $^2H_3$-epi-25(OH)D_3. Each serum sample was extracted using 1.5 mL hexane, and the supernatant was collected, dried under nitrogen at 25 °C, and reconstituted in 69 % methanol in water (volume fractions). Analytes were eluted from the extract (isocratic mobile phase same composition as the diluent) at a flow rate of 0.4 mL/min on a Hypersil GOLD pentafluorophenyl column (Thermo Fisher Scientific, Waltham, MA) at 28 °C and detected using APCI in positive-ion mode. Two transitions per vitamin D metabolite along with one transition per internal standard were monitored: 25(OH)D_3: *m/z* 383 → *m/z* 365 and *m/z* 383 → *m/z* 105; $^2H_6$-25(OH)D_3: *m/z* 389 → *m/z* 371; epi-25(OH)D_3: *m/z* 383 → *m/z* 365 and *m/z* 383 → *m/z* 105; $^2H_3$-epi-25(OH)D_3: *m/z* 386 → *m/z* 368; 25(OH)D_2: *m/z* 395 → *m/z* 377 and *m/z* 395 → *m/z* 209; $^2H_3$-25(OH)D_2: *m/z* 398 → *m/z* 380. Analytes were quantitated using six-point linear calibration curves traceable to SRM 2972 or SRM 2972a 25-Hydroxyvitamin D Calibration Solutions, and internal standards were used to correct for recovery.

Measurement of 25(OH)D_3 and 25(OH)D_2, by ID-LC-MS/MS (CDC Reference Measurement Procedure): Serum SRM 972a (500 µL) was accurately measured and spiked (gravimetrically) with pre-determined amounts of isotopically-labeled internal standards (ISTD), $^2H_6$-25(OH)D_3 and $^2H_3$-25(OH)D_2, to get an approximate 1:1 mass ratio of analyte to ISTD in glass tubes. The pH of each sample was adjusted to approximately pH 9.8 with aqueous sodium carbonate (0.1 g/mL), and the metabolites were extracted with hexanes twice. The combined extracts were dried under vacuum at 45 °C, reconstituted in 75 % methanol in water, vortex mixed, and filtered (0.45 µm PVDF, 96-well plate). Analytes were analyzed on LC-MS/MS with gradient elution (the ratio of methanol to water was gradually increased from 65 % to 80 % over 20 minutes) at a flow rate of 0.45 mL/min on a Acquity HSS PFP column (1.8 µm x 150 mm x 3.0 mm) (Waters Corp, Milford, MA) at 22 °C. APCI in the positive-ion mode and selected reaction monitoring (SRM) mode were used for detection with the following transitions: *m/z* 383 → *m/z* 365 (quantitation) and *m/z* 383 → *m/z* 105 (confirmation) for 25(OH)D_3; *m/z* 389 → *m/z* 371 for $^2H_6$-25(OH)D_3; *m/z* 395 → *m/z* 377 (quantitation) and *m/z* 395 → *m/z* 209 (confirmation) for 25(OH)D_2; and *m/z* 398 → *m/z* 380 for $^2H_3$-25(OH)D_2.

Measurement of 24R,25(OH)$_2$$D_3$ by ID-LC-MS/MS (NIST): Serum (1.5 g to 2.0 g) was spiked with an internal standard solution containing $^2H_6$-24R,25(OH)$_2$$D_3$. After equilibration at room temperature for 1 h, the pH of each sample was adjusted to pH 9.8 ± 0.2 with carbonate buffer. The 24R,25(OH)$_2$$D_3$ was extracted twice from the serum matrix with a mixture of hexane and ethyl acetate. The combined extracts were dried under nitrogen at 45 °C, and the residues were reconstituted with methanol for LC-MS/MS analysis. Extracts were analyzed using an Ascentis Express C_{18} column under isocratic conditions with a water:methanol mobile phase. APCI in the positive-ion mode and multiple reaction monitoring (MRM) mode were used. The following transitions were monitored: *m/z* 417 → *m/z* 381 for 24R,25(OH)$_2$$D_3$ and *m/z* 423 → *m/z* 387 for $^2H_6$-24R,25(OH)$_2$$D_3$.

Homogeneity Analysis: The homogeneity assessment was made at the time the certification analyses were performed. A stratified sampling plan was devised to test for homogeneity across the lot of vials. There was no apparent trend in the data when plotted against the sequence in which the vials were prepared, with the exception of 3-epi-25(OH)D_3 in Level 4. An additional component of uncertainty related to possible inhomogeneity has been included in the expanded uncertainty for this analyte in Level 4.

Certified Values for 25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3, and 24R,25(OH)$_2$$D_3$: The certified values for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 are based on the consensus of results from isotope dilution liquid chromatography mass spectrometry (ID-LC-MS) [3] and isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) [4] procedures performed at NIST, and from two different ID-LC-MS/MS procedures performed at the Centers for Disease Control and Prevention (CDC), Atlanta, GA [5,6]. The certified values for 24R,25(OH)$_2$$D_3$ are based on the results from a reference measurement procedure using ID-LC-MS/MS performed at NIST [7]. The NIST and CDC ID-LC-MS/MS methods are recognized as higher-order reference measurement procedures by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) [8,9].

The 25(OH)D_3 and 3-epi-25(OH)D_3 values are weighted means of the results from analyses at NIST using ID-LC-MS and ID-LC-MS/MS and from CDC using one ID-LC-MS/MS procedure. The 25(OH)D_2 values are consensus means obtained using the Bayesian hierarchical model in the NIST Consensus Builder [10] on the results from analyses at NIST using ID-LC-MS and ID-LC-MS/MS and from CDC using two ID-LC-MS/MS procedures. The 24R,25(OH)$_2$$D_3$ values are the results from analyses at NIST using ID-LC-MS/MS. The uncertainty provided with each certified value is an expanded uncertainty about the consensus mean to cover the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses and expresses both the observed difference between the results from the methods and their respective uncertainties, consistent with the Guide to the Expression of Uncertainty in Measurement and with its Supplement 1 [11−14]. 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 each analyte [11]. For the certified values shown in Table 1, $k = 2$.

Non-Certified Values for 25(OH)D_2 and 3-epi-25(OH)D_3: The non-certified values for 25(OH)D_2 and 3-epi-25(OH)D_3 are based on the agreement of results from ID-LC-MS and ID-LC-MS/MS procedures performed at NIST and from ID-LC-MS/MS results provided by the CDC. Values are weighted means of the results from analyses at NIST using ID-LC-MS and ID-LC-MS/MS and at CDC using ID-LC-MS/MS. The uncertainty provided with each non-certified value is an expanded uncertainty about the weighted mean to cover the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses and expresses both the observed difference between the results from the methods and their respective uncertainties, consistent with the ISO/JCGM Guide and with its Supplement 1 [11−14]. 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 each analyte [11]. For the non-certified values shown in Table A1, $k = 2$.

Certified and Non-Certified Values 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 values for total 25(OH)D, as the sum of the individual values for 25(OH)D_3 and 25(OH)D_2, are shown in Table 2 for certified values and Table A2 for non-certified values. The uncertainty provided with each value is an expanded uncertainty about the total 25(OH)D that covers the measurands 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 ISO/JCGM Guide and its Supplement 1 [11−14]. 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 analytes [11]. For the values shown in Table 2 and Table A2, $k = 2$.

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