



# Certificate of Analysis

## Standard Reference Material<sup>®</sup> 2973

### Vitamin D Metabolites in Frozen Human Serum (High Level)

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. A unit of SRM 2973 consists of one vial of frozen serum containing 25-hydroxyvitamin D [25(OH)D] and 24R,25-dihydroxyvitamin D<sub>3</sub> [24R,25(OH)<sub>2</sub>D<sub>3</sub>]. Measurement of 25(OH)D in serum, the sum of 25-hydroxyvitamin D<sub>2</sub> [25(OH)D<sub>2</sub>] and 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>], is generally considered a reliable indicator of vitamin D status. Measurement of 24R,25(OH)<sub>2</sub>D<sub>3</sub> in serum is considered as a catabolism marker and an indicator of kidney disease. The vial of SRM 2973 contains approximately 1 mL of serum.

For the majority of the U.S. population, serum concentrations for 25(OH)D typically range from 40 nmol/L to 75 nmol/L [1]. About 10 % of the population have 25(OH)D concentrations from 75 nmol/L to 120 nmol/L [1]. 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.

**Certified Values:** The certified value for 25(OH)D<sub>3</sub> is 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 [2]. The certified value for 25(OH)D<sub>3</sub> is based on results from the isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) procedure [3] performed at NIST. The NIST ID-LC-MS/MS method is recognized as a higher-order reference measurement procedure by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) [4].

**Reference Values:** Reference values for 25(OH)D<sub>2</sub>, 3-epi-25(OH)D<sub>3</sub>, and 24R,25(OH)<sub>2</sub>D<sub>3</sub> are provided in Table 2. A NIST reference value is a noncertified value that is 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 [2]. The reference values for 25(OH)D<sub>2</sub> and 3-epi-25(OH)D<sub>3</sub> are based on the results from ID-LC-MS/MS procedures performed at NIST. The reference values for 24R,25(OH)<sub>2</sub>D<sub>3</sub> are based on the results from a candidate reference measurement procedure using ID-LC-MS/MS performed at NIST [5].

**Expiration of Certification:** The certification of SRM 2973 is valid, within the measurement uncertainty specified, until **31 January 2023**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

**Maintenance of SRM Certificate:** NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Support for the development of SRM 2973 was provided in part by the National Institutes of Health (NIH) Office of Dietary Supplements (ODS). Technical consultation was provided by C.T. Sempos, J.M. Betz, and P.M. Coates (NIH-ODS).

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Overall direction and coordination of the analytical measurements leading to the certification of this SRM were performed by S. S.-C. Tai of the NIST Chemical Sciences Division.

Acquisition of the material was performed by K.W. Phinney of the NIST Biomolecular Measurement Division. Certification measurements were performed by S.S.-C. Tai. Additional measurements in support of the development of SRM 2973 were performed by M.A. Nelson, M. Bedner, B.E. Lang, M.M. Schantz, and L.T. Sniegoski of the NIST Chemical Sciences Division.

The NIST/NIH Vitamin D Quality Assurance Program (VitDQAP) at NIST was coordinated by M. Bedner. The value assignment of materials used for VitDQAP was performed by S.S.-C. Tai. The VitDQAP was used to evaluate the commutability of SRM 2973.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

## NOTICE AND WARNINGS TO USERS

SRM 2973 IS INTENDED FOR RESEARCH USE. 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 an 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/National Institutes of Health Manual [6].

## INSTRUCTIONS FOR STORAGE AND USE

**Storage:** Until required for use, SRM 2973 should be stored in the dark at a temperature between  $-20\text{ }^{\circ}\text{C}$  and  $-80\text{ }^{\circ}\text{C}$ .

**Use:** SRM 2973 contains one vial of frozen human serum. The vial 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.

## SOURCE, PREPARATION, AND ANALYSIS<sup>(1)</sup>

**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 and 24R,25(OH)<sub>2</sub>D<sub>3</sub> in SRM 2973 was based on the results from ID-LC-MS/MS measurements at NIST.

**Measurement of 25(OH)D<sub>3</sub>, 25(OH)D<sub>2</sub>, and 3-epi-25(OH)D<sub>3</sub> by ID-LC-MS/MS (NIST):** Serum (1.0 g to 2.0 g) was spiked with an appropriate internal standard solution (<sup>2</sup>H<sub>6</sub>-25(OH)D<sub>3</sub>, <sup>2</sup>H<sub>3</sub>-25(OH)D<sub>2</sub>, or <sup>2</sup>H<sub>3</sub>-3-epi-25(OH)D<sub>3</sub>). 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<sub>3</sub> and 3-epi-25(OH)D<sub>3</sub>;  $m/z$  407 →  $m/z$  389 for <sup>2</sup>H<sub>6</sub>-25(OH)D<sub>3</sub> and <sup>2</sup>H<sub>6</sub>-3-epi-25(OH)D<sub>3</sub>;  $m/z$  413 →  $m/z$  395 for 25(OH)D<sub>2</sub>; and  $m/z$  416 →  $m/z$  398 for <sup>2</sup>H<sub>3</sub>-25(OH)D<sub>2</sub>.

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<sup>(1)</sup> Certain commercial equipment, instruments or materials are identified in this certificate 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.

**Measurement of 24R,25(OH)<sub>2</sub>D<sub>3</sub> by ID-LC-MS/MS (NIST):** Serum (1.5 g to 2.0 g) was spiked with an appropriate internal standard solution (<sup>2</sup>H<sub>6</sub>-24R,25(OH)<sub>2</sub>D<sub>3</sub>). 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)<sub>2</sub>D<sub>3</sub> was extracted twice from the serum matrix with a mixture of hexane and ethylacetate. 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<sub>18</sub> 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)<sub>2</sub>D<sub>3</sub> and *m/z* 423 → *m/z* 387 for <sup>2</sup>H<sub>6</sub>-24R,25(OH)<sub>2</sub>D<sub>3</sub>.

**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.

**Certified Value for 25(OH)D<sub>3</sub>:** Value is the method mean of the results from analyses at NIST via a reference measurement procedure using ID-LC-MS/MS. The uncertainty provided with the 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 ISO/JCGM Guide [7]. The expanded uncertainty is calculated as  $U = k u_c$ , where  $u_c$  is the combined uncertainty and  $k$  is a coverage factor corresponding to approximately 95 % confidence for the analyte [7]. For the certified value shown in Table 1,  $k = 2$ . The measurand is the amount of 25-hydroxyvitamin D<sub>3</sub> listed in Table 1. Metrological traceability is to the SI derived unit for mass fraction (expressed as ng/g), mass concentration (expressed as ng/mL), and amount-of-substance concentration (expressed as nmol/L).

Table 1. Certified Value for 25(OH)D<sub>3</sub> in SRM 2973

	ng/g			ng/mL <sup>(a)</sup>			nmol/L <sup>(b)</sup>		
25-hydroxyvitamin D <sub>3</sub>	38.6	±	0.8	39.4	±	0.8	98.4	±	2.1

<sup>(a)</sup> The mass concentration level was calculated from the mass fraction using a measured serum density: 1.02229 g/mL.

<sup>(b)</sup> The molar concentration level was calculated from the mass concentration level using the relative molecular mass of 400.64 for 25(OH)D<sub>3</sub>. The equivalent conversion factor is 2.4960 for 25(OH)D<sub>3</sub>.

**Reference Values for 25(OH)D<sub>2</sub>, 3-epi-25(OH)D<sub>3</sub>, and 24R,25(OH)<sub>2</sub>D<sub>3</sub>:** Values are the method means of the results from analyses at NIST using ID-LC-MS/MS. The uncertainty provided with each reference 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 ISO/JCGM Guide [7]. The expanded uncertainty is calculated as  $U = k u_c$ , where  $u_c$  is the combined uncertainty and  $k$  is a coverage factor corresponding to approximately 95 % confidence for the analyte [7]. For the reference values shown in Table 3,  $k = 2$ . The measurands are the amounts of substance listed in Table 2 as determined by ID-LC-MS/MS. Metrological traceability is to the SI derived unit for mass fraction (expressed as ng/g), mass concentration (expressed as ng/mL), and amount-of-substance concentration (expressed as nmol/L).

Table 2. Reference Values for 25(OH)D<sub>2</sub>, 3-Epi-25(OH)D<sub>3</sub>, and 24R,25(OH)<sub>2</sub>D<sub>3</sub> in SRM 2973

	ng/g			ng/mL <sup>(a)</sup>			nmol/L <sup>(b)</sup>		
25-hydroxyvitamin D <sub>2</sub>	0.64	±	0.02	0.65	±	0.02	1.59	±	0.05
3-epi-25-hydroxyvitamin D <sub>3</sub>	2.05	±	0.08	2.10	±	0.08	5.23	±	0.20
24R, 25-dihydroxyvitamin D <sub>3</sub>	3.06	±	0.11	3.13	±	0.11	7.51	±	0.26

<sup>(a)</sup> Mass concentration levels were calculated from mass fractions using a measured serum density: 1.02229 g/mL.

<sup>(b)</sup> Molar concentration levels were calculated from mass concentration levels using the relative molecular masses. The relative molecular masses are 412.65 for 25(OH)D<sub>2</sub>, 400.64 for 3-epi-25(OH)D<sub>3</sub>, and 416.64 for 24R,25(OH)<sub>2</sub>D<sub>3</sub>. The equivalent conversion factors are 2.4234 for 25(OH)D<sub>2</sub>, 2.4960 for 3-epi-25(OH)D<sub>3</sub>, and 2.4002 for 24R,25(OH)<sub>2</sub>D<sub>3</sub>.

**Reference Value for Total 25(OH)D:** Vitamin D levels in serum are typically reported as the total of 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub>. The value for total 25(OH)D as the sum of the individual values for 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub> is shown in Table 3. 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 ISO/JCGM Guide [7]. The expanded uncertainty is calculated as  $U = k u_c$ , where  $u_c$  is the combined uncertainty and  $k$  is a coverage factor corresponding to approximately 95 % confidence. For the value shown in Table 3,  $k = 2$ . The measurand is the total 25(OH)D listed in Table 3 as determined by methods for 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub>. Metrological traceability is to the SI derived unit for mass fraction (expressed as ng/g) and mass concentration (expressed as ng/mL).

Table 3. Reference Value for Total 25(OH)D in SRM 2973<sup>(a)</sup>

	ng/g		ng/mL <sup>(b)</sup>	
Total 25(OH)D	39.2	± 0.8	40.1	± 0.8

<sup>(a)</sup> The value is denoted as a reference value based on the combination of a reference value for 25(OH)D<sub>2</sub> and a certified value for 25(OH)D<sub>3</sub>.

<sup>(b)</sup> The mass concentration level was calculated from the mass fraction using a measured serum density: 1.02229 g/mL.

**Commutability:** SRM 2973 was distributed as a blinded study material in the Summer 2014 comparability study of the 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<sub>2</sub> and 25(OH)D<sub>3</sub> metabolites, and the IA participants only reported values for total 25(OH)D [the sum of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>] in SRM 2973. Given that the concentration of 25(OH)D<sub>2</sub> is extremely low (Table 2) and below the limit of quantitation for most LC methods, the majority of the LC participants reported the same values for 25(OH)D<sub>3</sub> and total 25(OH)D. For the 63 values reported for total 25(OH)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 reference value of 40.1 ng/mL ± 0.8 ng/mL (Table 3) for total 25(OH)D. SRM 2973 is suitable for use with the majority of the methods used by VitDQAP participants.

## REFERENCES

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*Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail [srminfo@nist.gov](mailto:srminfo@nist.gov); or via the Internet at <http://www.nist.gov/srm>.*