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

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Standard Reference Material® 2971
24R,25-Dihydroxyvitamin D_3 Calibration Solution
CERTIFICATE OF ANALYSIS

Purpose: This Standard Reference Material (SRM) is intended for use in calibration of instruments and techniques used for the determination of 24R,25-Dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$].

Description: A unit of SRM 2971 consists of five two-milliliter ampoules of ethanolic solution. Each ampoule contains approximately 1 mL of solution.

Certified Values: The certified value for 24R,25$(OH)_2D_3$ is provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in accuracy in that all known or suspected sources of bias have been investigated or taken into account [1]. The certified value for 24R,25$(OH)_2D_3$ is based on results from the isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) procedure [2] and gravimetric preparation 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) [3].

The certified value for 24R,25$(OH)_2D_3$ is a weighted mean of the results from gravimetric preparation and the ID-LC-MS/MS. The certified mass fraction value (µg/g) and the concentration value (µmol/L) for 24R,25$(OH)_2D_3$ are provided in Table 1. The certified concentration value listed in Table 1 applies only to aliquots removed at 16 °C to 30 °C (see "Storage and Use").

Table 1. Certified Value for 24R,25$(OH)_2D_3$ in SRM 2971

	Mass Fraction[(a)] (µg/g)			Mass Concentration[(b)] (µmol/L)		
24R,25$(OH)_2D_3$	1.0544	±	0.0190	1.9936	±	0.0499

[(a)] The uncertainty provided is an expanded uncertainty (U) about the weighted mean to cover the measurand with approximately 95 % confidence. The expanded uncertainty is calculated as $U = ku_c$, where the combined standard uncertainty u_c incorporates the uncertainties and observed difference of values between the two methods, as well as the uncertainty of the purity assessment, and k is a coverage factor corresponding to approximately 95 % confidence. This approach is consistent with the JCGM and Supplement 1 [4–6]. Metrological traceability is to the International System of Units (SI) derived unit for mass fraction, expressed as microgram per gram, through purity assessment of neat calibrant material.

[(b)] The concentration value was obtained by multiplying the certified mass fraction value by the density of ethanol at 22 °C (0.78775 g/mL) and dividing by the relative molecular mass of 24R,25$(OH)_2D_3$ (416.64). This concentration value is for use in the temperature range of 16 °C to 30 °C, and an allowance for the change in density over this temperature range is included in the uncertainty.

Period of Validity: The certified values delivered by **SRM 2971** are valid within the measurement uncertainty specified until **30 April 2025**. 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 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 at the time of purchase. 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 free of charge through the NIST SRM website.

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Safety: SRM 2971 contains primarily ethanol, which is a flammable solvent. Open flames and sources of spark should be avoided while using this SRM. Use proper methods for disposal of flammable, potentially hazardous waste. Consult the Safety Data Sheet (SDS) for hazard information.

Storage: Sealed ampoules, as received, should be stored immediately in the dark at temperatures at –20 °C because of analyte instability at higher temperatures.

Use: Ampoules should be removed from the freezer and allowed to equilibrate to room temperature for 30 min under subdued light before weighing or volumetrically transferring. Precautions should be taken to avoid exposure of ampoules and test portions to strong UV light and direct sunlight.

Test portions for use should be withdrawn immediately after opening the ampoules and should be processed without delay for the certified values to be valid within the stated uncertainty. The certified concentration value listed in Table 1 applies only to aliquots removed at 16 °C to 30 °C.

REFERENCES

[1] Beauchamp, C.R.; Camara, J.E.; Carney, J.; Choquette, S.J.; Cole, K.D.; DeRose, P.C.; Duewer, D.L.; Epstein, M.S.; Kline, M.C.; Lippa, K.A.; Lucon, E.; Molloy, J.; Nelson, M.A.; Phinney, K.W.; Polakoski, M.; Possolo, A.; Sander, L.C.; Schiel, J.E.; Sharpless, K.E.; Toman, B.; Winchester, M.R.; Windover, D.; *Metrological Tools for the Reference Materials and Reference Instruments of the NIST Material Measurement Laboratory*; NIST Special Publication (NIST SP) 260-136, 2021 edition; U.S. Government Printing Office: Washington, DC (2021); available at https://nvlpubs.nist.gov/nistpubs/SpecialPublications/NIST.SP.260-136-2021.pdf (accessed Mar 2022).

[2] Tai, S.S.-C.; Nelson, M.A.; *Candidate Reference Measurement Procedure for the Determination of 24R,25-Dihydroxyvitamin D3 in Human Serum using Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry*; Anal. Chem., Vol. 87, pp. 7964-7970 (2015).

[3] Joint Committee for Traceability in Laboratory Medicine; available at https://www.bipm.org/en/committees/jc/jctlm/ (accessed Mar 2022).

[4] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (2008); available at https://www.bipm.org/en/publications/guides (accessed Mar 2022); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at https://www.nist.gov/pml/nist-technical-note-1297 (accessed Mar 2022).

[5] JCGM 101:2008; *Evaluation of Measurement Data — Supplement 1 to the "Guide to the Expression of Uncertainty in Measurement" — Propagation of Distributions Using a Monte Carlo Method*; JCGM (2008); available at https://www.bipm.org/en/publications/guides (accessed Mar 2022).

[6] Efron, B.; Tibshirani, R.J.; *An Introduction to the Bootstrap*, Chapman & Hall, UK (1993).

Certificate Revision History: 16 March 2022 (Change of period of validity; updated format; editorial changes); 05 March 2019 (Update to "Instructions for Storage and Use"; editorial changes); 26 February 2018 (Original certificate 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

PREPARATION AND ANALYSIS

The solution was prepared gravimetrically at NIST from anhydrous ethanol and the primary standard for 24R,25(OH)$_2$D$_3$ obtained from IsoSciences (King of Prussia, PA). The solution was stirred for 90 min after preparation and then stored at 4 °C overnight. The morning following preparation, the solution was removed from the refrigerator and stirred for 30 min. The solution was then chilled completely with ice and then aliquoted into 2 mL amber glass ampoules that had been purged with argon prior to addition of the solution. The ampoules were then flame-sealed. The mass of the primary standard and the total mass of the solution were used to calculate the gravimetric concentration.

Measurement of 24R,25(OH)$_2$D$_3$ by ID-LC-MS/MS (NIST): One ampoule from each of 23 boxes across the lot of ampoules was randomly selected for analysis. An aliquot approximately 200 µL from each ampoule was spiked gravimetrically with an internal standard solution [24R,25(OH)$_2$D3-d_6] to get an approximately 1:1 mass ratio of analyte to internal standard. Each sample was mixed thoroughly, dried under nitrogen at 45 ºC, and the residue was reconstituted with methanol for LC-MS/MS analysis. Samples 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 24R,25(OH)$_2$D$_3$-d_6.

Homogeneity Analysis: The homogeneity assessment was made at the time the certification analysis was conducted using the ID-LC-MS/MS method. One ampoule from each of the 23 boxes across the production lot was randomly selected for analysis. A small uncertainty component was incorporated in the uncertainty of the weighted mean to account for a minor variation in filling across the production lot.

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APPENDIX B

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.

Support for the development of SRM 2971 was provided in part by the National Institutes of Health (NIH) Office of Dietary Supplements (ODS). Technical consultation was provided by S.A. Wise, C.T. Sempos, J.M. Betz, and P.M. Coates (NIH-ODS).

Certification measurements were performed by S.S.-C. Tai. Additional measurements in support of the development of SRM 2971 were performed by M.A. Nelson and B.E. Lang of the NIST Chemical Sciences Division.

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.

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